IDENTIFYING FAVORABLE RESPONSES TO DROUGHT STRESS IN DIVERSE GENOTYPES OF MAIZE (*ZEA MAYS* L.)

A Thesis

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ABSTRACT

Of the many potential abiotic stresses, insufficient water supply is the major limiting physiological constraint for crop production in many regions of the world. Because yield is a product of many phenotypic components, plant breeders experience difficulties when selecting lines for higher yield under drought conditions. The use of other measurable traits, such as reproductive timing or concentrations of stress hormones, can reveal information regarding changes in plant behavior during low water availability. The purpose of this project is to further decipher the signaling behind the drought response of maize and identify which physiological responses might characterize drought-resistant genotypes. Eight tropical inbred maize genotypes, previously shown to have varying behavior under drought conditions, were surveyed for physiological response during water stress. Water stress was imposed once plants reached the flowering period. During a ten-day treatment period, transpiration, ear growth and silk length were calculated daily for each plant. Upon tissue harvest (after ten days of treatment), ear size and leaf water potential were measured. Leaf and ear tissue sampled in the greenhouse were analyzed for non-structural carbohydrates, abscisic acid (ABA), cytokinin, and invertase. Physiological differences in genotypic response to water stress were apparent. Of the eight lines examined, the P1 genotype appeared to respond best to low water availability. Under water stress, P1 maintained low ear and leaf ABA concentrations, higher sucrose levels in the leaves, more starch in the ear tissue, and exhibited little change in ovary invertase activity. When comparing measured traits among all genotypes, leaf and ear ABA was negatively correlated with reproductive growth. Leaf carbohydrate levels were positively correlated with ear growth, and negatively associated with ABA levels in the leaves and ears. In general, cytokinin levels in the ear tissue increased under water stress.

Invertase activity decreased in water-stressed ear tissue compared to control plants, and cell wall invertase activity was higher than soluble invertase. Carbohydrate partitioning appears to be an important indicator of reproductive growth under water stress, and identifying the differences in invertase behavior and their relationship to yield performance in several genotypes would be a crucial next step in assessing drought tolerance.

BIOGRAPHICAL SKETCH

Amanda grew up on a corn-and-soybean farm in central Illinois, where she regularly played in the dirt with her three sisters and cheered on the Chicago Cubs. Beginning with her earliest memories, her grandfather, Paul Solliday, helped establish her fascination with the outdoors.

As a high school student, Amanda took as many science classes as would fit in her schedule. While attending Illinois Wesleyan University, Amanda decided to major in both biology and environmental studies. A semester abroad at King's College in London helped remove any of her xenophobia. In her spare time, Amanda worked on various research projects including pesticide contamination in wildlife, soybean pollination, maize genomics, organic farming methods, a state-wide nitrogen survey, and insect evolutionary development.

Her interests in the interplay between the natural environment and agriculture led her to Cornell University. It's been a good journey, and she is excited for the next step.

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CHAPTER 1

INTRODUCTION

Water scarcity is becoming an increasingly salient issue as the world population continues to grow and climate becomes increasingly more erratic (IPCC, 2007). Agricultural production already consumes seventy percent of anthropogenic withdrawals from freshwater resources (Molden, 2007). Of the many potential abiotic stresses, insufficient water supply is the major limiting physiological constraint for crops in many areas. According to the International Maize and Wheat Improvement Center (Listman *et al*., 2005), drought is responsible for approximately fifteen percent of maize yield losses worldwide. This yield loss represents around 200 million tons, roughly the annual maize production of Mexico. The threat of yield loss due to drought is even more severe in developing countries of the tropical regions, where rainfall can be erratic and poverty is most prevalent.

Period of Drought Susceptibility

From a maize farmer's perspective, the most devastating time for water stress to occur is the flowering period (Salter & Goode, 1967; Grant et al., 1989). The reproductive organs are more vulnerable at this time than during earlier stages of vegetative growth or the later grain-filling stage. Environmental stress decreases photosynthetic assimilation, and during the flowering or reproductive period maize responds to this stress by decreasing the energy it expends towards reproduction. The kernels of a maize plant can constitute half the shoot dry weight at physiological maturity. A maize plant subjected to drought stress during the flowering period can lose all kernel mass, with the plant surviving yet completely barren (Duvick *et al*., 2004).

Around the time of pollination, water deficit causes a decrease in kernel number. At this time, reserves in the parental plant cannot sustain ovary growth. Later in maize reproductive development (during grain fill) water stress reduces kernel size while the number is maintained. A reduction in kernel size can be offset by remobilization of biomass from the stems and leaves, and yield losses tend to be less severe during this later stage (Boyer & Westgate, 2004). Because of the increased vulnerability to water stress during early stages of reproductive development, this stage of the plant's life cycle is an important target in breeding for drought tolerance.

When exposed to water stress, maize can differ widely in ear and silk growth rates due to genotypic differences. Many breeding efforts to reduce drought susceptibility involve selection for a reduced anthesis-silking interval. A longer anthesis-silking interval, or a greater degree of asynchrony in male (anthesis) and female (silking) reproductive development, has been associated with low yield in maize. Timing of anthesis is not altered by water deficit, but silk emergence is generally delayed (Monneveux & Ribaut, 2006; Welcker *et al*., 2007). When subjected to drought stress, silks can emerge too long after anthesis to be effectively pollinated. Silks lose receptivity to pollen as they age, and drought stress can even further decrease the window of time for receptivity. Therefore, early silk emergence is one desirable breeding characteristic for drought tolerance in maize (Boyer & Westgate, 2004).

Photosynthate Status under Water Deficit

During water shortages, due to diminished photosynthesis, the phloem delivers less sugar to the pedicel terminus of the ovaries. These translocated photosynthates are primarily in the form of sucrose. The phloem does not transport sucrose directly to the developing ovaries. Phloem tissue terminates in the pedicel, except for a small number

of vascular bundles which travel to the silks. Two possible pathways are responsible for the cleavage of sucrose at the phloem termini – the invertase reaction or the reversible sucrose synthase pathway (Sturm & Tang, 1999). At the ovary, these enzymes control sugar uptake by the ovaries. In the post-phloem tissue of the pedicel, sucrose is cleaved into glucose and fructose (hexoses) at the phloem termini. Hexoses enter the nucellus via post-phloem diffusive transport. During water shortages, less sugar arriving from the leaves results in a decreased gradient of hexoses, inhibiting movement into nucellus (via diffusion) (Boyer & McLaughlin, 2007). For maize reproductive development during the flowering period, the invertase reaction is more important for transfer of sugars to the developing kernels than the sucrose synthase pathway (Zinselmeier *et al*., 1995b).

In maize kernels, two main forms of invertases are present – cell wall and soluble (Xu *et al*., 1995). During water stress, both soluble and cell wall invertase activity appear to be reduced. Down-regulation of invertase occurs during water stress periods and causes a reduction in sucrose metabolism in the maize kernels (Zinselmeier *et al*., 1999). Soluble invertase in the maternal tissue of the nucellus seems to be the most crucial form of the enzyme for sucrose metabolism during early reproductive development prior to fertilization (Andersen *et al*., 2002; Jain *et al*, 2008). Cell wall invertase, located in the basal endosperm, becomes more important later in maize kernel growth after pollination (Chourey *et al*., 2006; Jain *et al*, 2008). However, both forms have been found to be active in prepollination kernels (Zinselmeier *et al*, 1999; McLaughlin & Boyer, 2004).

Zinselmeier *et al.* (1999) showed that sucrose feeding to maize stems can rescue kernels destined to abort and restore starch stores and invertase activity in kernels.

Accordingly, sucrose may serve as a signal, as well as a substrate. Two sugar signals are sent from the ovaries, the first when a decrease in sucrose occurs due to slower delivery from the phloem and a second when glucose levels decrease as starch is consumed during the deficit (McLaughlin & Boyer, 2004). The most important sugar status signal is believed to be low sucrose in the kernel, because down-regulation occurred before glucose was depleted. Sucrose fed to stems can prevented abortion of ovaries. While a larger pool of reserves in the plant as a whole may not help to maintain ovary growth during water deficit, local sugar status of ovary tissues appears to be important (Boyer & McLaughlin, 2007). Interestingly, Setter *et al*. (2001) reported *higher* carbohydrate levels in the ears of water-stressed plants, which suggest that the signal is not simply local sugar status.

Reserves in the kernel are present primarily in the form of starch, but also as sucrose. When present in excess, plants can convert hexoses to starch reserves. During periods of water stress, plants utilize these same reserves. While stress diminishes or eliminates supply of sugar from photosynthesis, respiration continues to require substrate to sustain cellular activities. Starch is stored in ovary walls or converted to ovary constituents for development and provides a buffer for depletion of glucose levels. When ovaries are destined to abort, starch is first utilized, followed by sugars (Boyer & McLaughlin, 2007). This process of remobilization of resources is common in many plants. On a smaller scale, this process of utilizing storage reserves occurs in the leaves every night as plants can not photosynthesize in the dark. Starch synthesized during the day in the leaves is converted to sucrose for phloem transport (Trouverie & Prioul, 2006).

Maintenance of Water Potential and Osmotic Adjustment

Due to its C4 metabolism, maize has relatively high water use efficiency, or a high photosynthetic rate with a corresponding low transpiration rate. Maize is also isohydric, meaning that it can maintain leaf water potential at high values under water stress due to stomatal closure. This characteristic helps avoid leaf dehydration during periods of low water availability (Welcker *et al*., 2007).

Changing solute potential is another important growth process underlying drought tolerance. Low water potential and associated turgor loss cause a reduction in expansive growth through turgor loss. Plants can prevent damage due to low water potential by accumulating osmotically active solutes like sugars and salts to maintain turgor and expansive growth (Morgan, 1984). Turgor maintenance can vary among maize genotypes (Bouchabke *et al*., 2006) and parts of the plant (Westgate & Boyer, 1985). However, Boyer & McLaughlin (2007) suggest that abortion of kernels in maize is a response to sugar nutritional status and not osmotic potential, based on the low water stress response other osmolytes, such as amino acids and salts. But sugars can serve as osmotically active solutes in addition to substrates for cellular processes, and Chimenti *et al*. (2006) found that osmotic adjustment during the flowering period can improve maize yields in temperate genotypes, and additionally conveys no yield penalty under well-watered conditions. Bolanos and Edmeades (1991) conducted a survey of 204 tropical genotypes and found that a small percentage (7%) was able to adjust osmotically when subjected to water stress and significant variation in constitutive solute potential levels among genotypes was observed. Due to the potential for improving maize yields and variation present among genotypes, osmotic adjustment appears to be a useful trait to study when examining drought tolerance.

Drought Signaling via Hormones

In addition to sugar signaling, the photosynthate status may also be signaled via hormones, such as abscisic acid (ABA) and cytokinin. Hormones are efficient messengers, as they travel long distances and therefore can induce a systemic response instead of a small, localized reaction. Increased ABA levels in the leaf tissue cause the stomates to close. The reduction in stomatal conductance increases water use efficiency of the plant, and therefore increases the ability of the plant to tolerate water stress. However, stomatal closure inhibits the plant's ability to photosynthesize and this affects reproductive development (Zhang and Davies, 1990; Zinselmeier *et al*., 1999; Boyer & Westgate, 2004; Liu *et al*., 2005). In maize plants, many studies have also reported increased ABA concentrations in maize florets during water deficits around the pollination period (Zinselmeier *et al*., 1999; Setter *et al*., 2001). ABA may inhibit cell division and cause abortion in the kernel during post-pollination (Mambelli & Setter, 1998; Setter & Flannigan, 2001).

Cytokinin is another hormone that may be involved in drought signaling. Many studies have shown cytokinin regulation of kernel development (Brugiere *et al*., 2003; Jones & Setter, 1993). Higher levels of cytokinin result in an increase of lateral root meristem activity and in developing maize kernels are associated with high rates of cell division (Bilyeu *et al*., 2003). Furthermore, cytokinin has been associated with prevention of stomatal closure in leaves (Vysotskaya *et al*., 2004) and is believed to be antagonistic to ABA (Davies *et al*., 2005). Reductions in cytokinin oxidase, the enzyme that degrades cytokinin, cause an accumulation of cytokinin which increases the number of reproductive organs in rice (Ashikari *et al*., 2005). Under normal conditions, cytokinin oxidase regulates growth and development in maize kernels and can even be induced by ABA under stress conditions. In maize kernels, mRNA levels

of *Ckx1,* the gene encoding cytokinin oxidase, were upregulated during drought conditions (Brugiere *et al*., 2003). The relationship between cytokinin, kernel development, and transpiration makes it a likely candidate for involvement with ear growth under water stress conditions.

Rationale and Objectives of this Study

Selective breeding has improved the drought response of maize and reduced the impacts of water stress on yield (Monneveux et al., 2006). Furthermore, work with transgenic drought tolerance solely has shown little success in the field, emphasizing the importance of a traditional breeding approach to drought tolerance (Marris, 2008). Because yield is a product of many phenotypic components (such as kernel number, ear size, grain weight, etc.), it is difficult to efficiently select lines for higher yield under drought conditions because of the many possible sources of variability. The use of other measurable traits, such as the continuation of ear growth/abortion decisions or the concentration of hormones activated during stress conditions, can reveal information about how the plant behavior underlying yield may change under water-stressed conditions. These traits are potentially more manageable than yield for plant breeders due to their greater heritability and stability (Monneveux & Ribaut, 2006).

In the continual search for higher yields under less-than-ideal environmental conditions, it is important to understand the mechanisms underlying reproductive growth. Imperceptible changes in physiology can occur before anything is noticeable visually. The purpose of this project is to further decipher the signaling behind the drought response of maize and identify which physiological responses might characterize drought-resistant genotypes. To do this, I have examined reproductive growth in maize under water deficit, as well as carbohydrate partitioning and the

behaviors of two plant hormones, abscisic acid (ABA) and cytokinin. Each potentially provides useful information for determining the processes underlying maize reproductive success (i.e., high yields) under water stress during the flowering period.

Because of the evidence showing ABA, cytokinin and sugar signaling in water-stressed conditions, I hypothesize that for maize subjected to drought at flowering, a genotypic relationship exists between the decision of the ear to sustain growth/abort and cytokinin, ABA and invertase concentration in the ears. I predict that in plants with higher cytokinin concentrations and invertase activity under drought conditions, ears will be less likely to abort. ABA will have the opposite effect, and plants with high ABA levels will have less reproductive success. Genotypes able to maintain high invertase activity in the ears, and therefore sugar supply to the developing kernels, will also exhibit higher reproductive success.

By elucidating the roles of plant hormones and enzymes in maize under water stress during the sensitive flowering period and their relationship to ear growth decisions, another effective tool can be developed to combat crop damage from drought. If a correlation exists between cytokinin, ABA or invertase and changes in ear growth due to water stress, this information will be the first step in identifying candidate genes for parental lines used in selective breeding for improved yield under drought stress.

To strengthen the characterization of water deficit response in maize, my project will examine multiple genotypes. Due to interest in the particularly susceptible flowering period, this project focuses on the pre-pollination period of maize reproductive development. Developing a more encompassing picture of the events occurring in lines with superior and inferior drought response can be used to target desirable genes for breeding efforts to maintain reproductive growth in maize under periods of low water availability.

CHAPTER 2

MATERIALS AND METHODS

Plant Material

Eight tropical inbred genotypes were used in the first two experiments (Table 1). The maize lines chosen for this experiment previously showed varying response to drought in CIMMYT breeding trials (Messmer, 2006; CIMMYT, 2005; Ribaut *et al*., 2004). Abbreviated names will be used in results and discussion section.

Table 1. List of genotypes surveyed and anticipated response to water stress.

Abbreviated Name	**Full Name**	**Expected Drought Response**
247	CML247	Susceptible
312	CML312	Susceptible
444	CML444	Tolerant
H16	H16	Varied
K64	K64R	Varied
Malawi	SC-Malawi	Susceptible
P1	Ac7643	Tolerant
P2	7729/TZSRW	Susceptible

The purpose of Experiment I was to characterize our experimental set-up. All eight genotypes were surveyed but sequential planting only allowed comparison between treatments, and not among genotypes. Experiment II was designed to determine genotypic differences in drought response. All eight genotypes were examined, but P2 was removed from analysis due to disease susceptibility. In Experiment III, only P1 (drought-tolerant) and P2 (drought-susceptible) genotypes were studied.

Growth Conditions

Seed was sown in 10-liter pots with drain-holes (Poly-tainer #3, Nursery Supplies Inc., Chambersburg, PA, USA) containing Cornell Plant Breeding rooting medium (vermiculite:sphagnum peat moss [3:2 volume ratio], 4 g L^{-1} powdered dolomitic limestone and 3.2 g L^{-1} of Peter's Unimix Plus III 10-5-10 fertilizer [Scotts Company, Marysville, OH, USA]) to which 35 g L^{-1} of CaSO4 and 42 g L^{-1} of powdered FeSO4 were added and thoroughly mixed. An automated system watered pots each day with 0.5 to 1.0 liter (depending on plant size) of nutrient solution containing 0.6 g L^{-1} of Peters 15-5-15 Ca-Mag (Scotts Co., Marysville OH). Additional water was manually supplied to meet transpiration requirements and leach excess salts.

Plants were grown in a greenhouse at Cornell University located in Ithaca, New York (latitude 42.42N). Artificial illumination was provided by 1000 W metal halide lamps for a 12-h light period. In Exp. I (April 23 to June 6, 2007), day/night temperature averaged 28/22°C and photosynthetically active radiation (PAR) (400-700 nm) averaged 36.6 mol/(m^2 d). In Exp. II (July 17 to September 16, 2007), day/night temperature averaged 28/22°C and PAR averaged 32.2 mol/(m^2 d). In Exp. III (February 10 to March 15, 2008), day/night temperature averaged 25/21°C and PAR

averaged 15.5 mol/(m^2 d). Plant density was approximately six plants per square meter.

Low Ψ_w Treatments

High water potential (Ψ_w) was maintained until plants reached the flowering period. Timing of treatment initiation (entry into experiment) varied slightly among the three experiments, although all experiments imposed treatments during the flowering period. Treatments were initiated in the first experiment when the tassel of the plant was fully extended, in the second experiment when the silks had grown to a length equal to the entire length of the ear, and in the third experiment when the silks had grown three to five centimeters past the ear tip. At treatment initiation, silks had not yet emerged from the husk in any of the three experiments. Upon entry into the experiment, irrigation/nutrient applications were suspended. Plants were then manually watered according to treatment. Water-stressed and well-watered control treatments were randomly assigned to each pot. Controls were watered to soil capacity each day to maintain high Ψ_w. In Exp. I and Exp. II, the water-stressed treatment received 30% of the volume transpired by the controls, after an initial drying phase. In Exp. III, stressed plants were allowed to dry down to a gravimetric set point that was 40% by mass of total pot capacity when well-watered. After reaching the critical mass, plants were watered each day to return to 40% of total pot capacity. Daily transpiration was measured gravimetrically.

Greenhouse Measurements and Sampling (Phenotypic Assessment)

Ear shoots were bagged prior to silk emergence. The husks were cut back to reveal ear tissue for growth measurements. Each day, ear and silk growth were measured for each plant. Leaf tissue was also sampled daily (2 discs, each approximately 7 mm in

diameter) and immediately transferred to chilled 80% (v/v) methanol for ABA and carbohydrate analysis. Dates of reproductive events (anthesis and silking) were recorded for each plant.

In Exp. III, non-manipulated controls were included to check for surgical effects resulting from cutting back the husk tissue. No effects from surgery were observed. Also, approximately half of the plants were pollinated and allowed to grow to full maturity before harvesting to obtain a realistic yield estimate for both the control and water-stressed treatment. One day before pollination, plants were re-watered to ensure successful pollen transfer to the silks. Thirty days after pollination, kernel number and dry mass were determined.

Upon tissue harvest (10 days after treatment initiation), leaf senescence was scored. Final length, diameter and mass measurements were recorded for the ear. Leaf and ear total water potential were determined at harvest using a thermocouple psychrometer with nanovoltmeter (SC-10, Decagon Devices, Inc., Pullman, WA). The psychrometer was calibrated with 0.1 to 0.5 molal range of potassium chloride solutions using methods similar to those described in Melkonian *et al*. (2004). After leaf and ear total water potential were measured, tissue was frozen, crushed with pliers to exude sap, and reassembled in the psychrometer to ascertain solute potential. Leaf water potential was also measured using a pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA), according to methods described by Boyer (1995). The apical regions of the ears were immediately frozen in liquid nitrogen at -196°C for further laboratory analysis.

Laboratory Analysis

Leaf and ear tissues that were sampled in the greenhouse were analyzed for non-structural carbohydrates, ABA, cytokinin, and invertase. Upon sampling in the greenhouse, leaf tissue was stored in chilled 80% methanol and ear tissue was frozen with liquid nitrogen. Before physiological quantifications, the frozen ear samples were ground to a fine powder with a mortar and pestle chilled with liquid nitrogen.

Carbohydrates

Sucrose, glucose and starch were measured for both ears and leaves using the peroxidase/glucose oxidase (PGO) method similar to Ober *et al*. (1991) and Setter *et al*. (2001). The PGO method is based on the Trinder reaction, where glucose reacts with O_2 (catalyzed by glucose oxidase) to form gluconic acid and H_2O_2. Catalyzed by peroxidase, the H_2O_2 immediately reacts with *p*-hydroxybenzoic acid and 4-amino-antipyrine to create a bright pink dye complex (Trinder 1969).

Crude extract was used for leaf samples analyzed for sucrose and glucose content. An intermediate dilution was performed for ear samples to ensure that carbohydrate levels were on-scale for reading by the spectrophotometer. Dried samples were redissolved in a known volume of 0.01% azide water, and an aliquot was transferred to 96-well plates containing 50 µl autoclaved water. To analyze glucose content, 150 µl of PGO solution (peroxidase and glucose oxidase enzymes in buffer solution containing 100 mM KH_2PO_4-NaOH (pH 7.0), xxx mM para-hydroxybenzoic acid, yyy mM 4-aminoanti pyrene, 0.1% (w/w) bovine serum albumin, and 0.01% sodium azide) were added to each well. After full color development at room temperature, the plates were read on a Packard SpectraCount model 750 spectrophotometer (490 nm wavelength setting). To quantify sucrose content, an invertase solution (292 U/mg, 10mg/mL H_20)

was added to the samples, and reaction was allowed to run until full color development of sucrose standards before reading on the spectrophotometer (490 nm). Standards made from dilutions of glucose (3.2 mg/mL) and sucrose (2.5 mg/mL) solutions were used to calibrate the assay.

After all free sugars were extracted, starch content was also determined. After samples were dried overnight, each sample was rediluted in 200 µL azide water, covered, and incubated at 80°C to gelatinize starch. After two hours, samples were cooled and 200 µL enzyme solution (250 mM acetate buffer at pH 4.5, 74 U/mg amyloglucosidase, 20 U/mg α-amylase, 0.1% w/v sodium azide, and 0.1% BSA) were added to hydrolyze starch into glucose. The reaction was incubated for two days on a rotary shaker at 37°C. Samples were then stored in 5°C. The PGO method was then used to determine the amount of glucose cleaved from starch.

Chromatography

Prior to hormone analysis, both leaf and ear tissue were first separated into fractions based on hydrophobicity using reverse phase C_{18} chromatography, modified from Ober et al. (1991) and Setter et al. (2001). Supelco columns (DSC-18 SPE-96) with 25 mg of C_{18} packing material were used in a 96-well vacuum apparatus. Columns were washed with 95% ethanol and 30% methanol prior to use. Extracts from samples stored in 80% methanol were transferred to a 96-well plate, dried in a forced-air incubator at 45°C, then redissolved in 100 µL 30% MeOH and 1% v/v glacial acetic acid with 20 µL 0.04% bromecresol green added as a chromatograph tracer. Samples were loaded onto the columns with 120 µL 30% methanol, and pulled through by vacuum. Columns were then washed with 200 µL 30% methanol to remove any remaining hydrophilic compounds. Abscisic acid was eluted from the columns using

200 µL 65% methanol with 1% acetic acid, followed by a 200 µL 95% ethanol to remove any lingering compounds. NH_4OH was added to neutralize the acetic acid. Plates were read on a spectrophotometer (Packard SpectraCount model 750) using a 590 nm wavelength to measure bromecresol green. Chromatographic yield exceeded ninety-five percent, so no adjustment for losses was necessary.

For the ear tissue of Exp. III only, RP-CE Strata-X-C columns (33µm 30mg) columns were used for chromatography. The same procedure was followed as that of the C_{18} chromatography above, with a few adjustments. Eight total fractions were collected, with solvents for each as follows: 1) 30% MeOH + 0.2M formic acid, 2) 30% MeOH + 0.2M formic acid, 3) 30% MeOH + 0.2M formic acid, 4) 65% MeOH + 0.2M formic acid, 5) 65% MeOH + 0.2M formic acid, 6) 65% MeOH + 0.35M NH_4OH, 7) 65% MeOH + 0.35M NH_4OH, 8) 95% EtOH.

Hormones (Abscisic Acid and Cytokinin)

ABA and cytokinin levels were determined using an enzyme-linked immunosorbant assay (ELISA) as described in Setter *et al*. (2001). The 65% methanol fractions from reverse phase C_{18} chromatography were used for ABA quantification, and load and wash 30% methanol fractions combined for the cytokinin assay. After drying, samples were redissolved in 100 µL azide water (0.01%).

For both hormones, 96-well plates were coated overnight with a BSA conjugate solution (ABA-BSA for abscisic acid, ZR-BSA for zeatin) containing 1.4 µg BSA conjugate per plate and 50mM $NaHCO_3$ at a pH of 9.6. Plates were then washed four times with a TBS (10mM tris-hydroxymethyl aminomethane-HCl, pH of 7.5, 1 mM $MgCl_2$, 100mM NaCl) and 0.1% Tween-20 solution (TBST). Dried samples were

redissolved in 200 µL of water (containing 0.01% azide) and 20 µL was dispensed into 90 µL of MBSA (50mM MOPS-NaOH, pH 7.5, 1 mM $MgCl_2$, 100 mM NaCl) and 100 µL of primary antibody solution (100 µL MBSA containing 1 µg of anti-ABA or anti-ZR monoclonal antibody) (Setter *et al*., 2001). A calibration curve was generated using a serial dilution of ABA or Z/ZR standards ranging from 0.002 to 5 pmol/well. After incubating overnight at 5°C, the plates were again washed four times with a TBST solution. Secondary antibody solution (200 µL containing 10 nL of anti-mouse IgG-alkaline phosphatase (reporter enzyme) conjugate in MBSA) were added to each well. Reaction was run overnight at 5°C. After washing four times with a TBST solution, 200 µL PNPP (0.2 mg *p*-nitrophenyl phosphate in 0.9M diethanolamine and 3 mM $MgCl_2$ at pH 9.8) substrate solution was added and the reaction incubated for 60 min at room temperature before reading absorbance and 405 nm with spectrophotometer (Packard SpectraCount model 750).

Invertases

Soluble and cell wall acid invertase activities (sucrose hydrolysis) were examined in ear tissue only. A buffer solution containing 50 mM Hepes-KOH (pH 7.4) buffer (tissue:buffer; 1:2(w/v)) containing 5 mM $MgCI_2$, 1 mM EGTA, 1 mM EDTA, 40% (v/v) glycerol, 0.1% BSA, 0.5 mM DTT and 2% PVP was added to sample tissue in 1:2 tissue:buffer w/v and stored at -18°C.

To determine soluble invertase activity, the samples were centrifuged at 14,000 rpm for 10 minutes and the supernatant was removed and transferred to separate Eppendorf tubes. Sugars were removed from the sample solution using a waterbug dialysis method (Orr *et al*. 1995). Caps and sealing rings were cut from Eppendorf tubes. The soluble invertase ear samples (100 µL) were dispensed into the cap. Dialysis tubing

(molecular weight cut off 12,000-14,000) was placed over the cap and secured by pressing down the sealing ring. The units were then placed in a 10 mM acetate buffer (pH 4.5) with the tubing in contact with the buffer solution. Dialysis ran overnight at 24°C. 50 µL of the samples were dispensed into a 96-well reaction plate containing 150 µL of 37.5 mM acetate buffer and 0.25 M sucrose in each well. After dispensing all the samples into the reaction plate, an aliquot was taken to establish the native glucose present in the solution (t=0). Aliquots were dispensed into 50 µL of 0.02 M NaOH to stop the enzymatic reaction by altering the pH. Incubation lasted 6.5 hours, with four time points taken at t=0, t=30, t=180 and t=390. Reactions were linear with time.

The leftover tissue was washed twice with 1 mL 50mM Hepes-KOH to remove native sugars, centrifuging and removing supernatant between washes. Cell wall invertase activity was ascertained using the remaining pellet of ear tissue. To begin the enzyme reaction, 150 µL of 50mM acetate buffer (pH 4.1) with 50 uL of 1 M sucrose was added to each tube and mixed vigorously. An aliquot was taken immediately after addition of the reaction solution, to establish a glucose level for time zero (t=0). The reaction was incubated for five hours at room temperature, with four time points taken at t=0, t=45, t=180 and t=300. The reactions were linear with time.

Glucose product for both soluble and cell wall acid invertase reactions were quantified using the PGO method, previously described in the carbohydrate section.

Statistical Analyses

Plants were randomly assigned to watering treatments. Significance of differences in variable means was determined by ANOVA with standard least squares values using

JMP software (SAS Institute, 2008). Data were log-transformed for analysis to fit normal distribution, if necessary. Correlations were performed with Excel Analysis ToolPak using trait averages for each genotype (Microsoft, 2003).

CHAPTER 3

RESULTS

Experiment I

To characterize the effects of water stress for our greenhouse experimental set-up, comparisons between treatments were first examined. Measurements taken in the greenhouse included daily silk growth, final leaf water potential, and final leaf senescence. Water-stressed plants had reduced silk growth compared to well-watered controls (Figure 1.1), and water deficit reduced the ear dry mass at the conclusion of the experiment (Figure 1.2). Leaf water potential was lowered to -1.3 MPa in water-stressed treatments during predawn conditions, compared to -0.4 MPa for controls (Figure 1.3). For water-stressed plants, leaf senescence averaged 7 leaves per plant, while controls had 2 senesced leaves on average (Figure 1.4).

Ear tissue was harvested at the conclusion of the ten day treatments, and abscisic acid, cytokinin, and leaf sugars were quantified in these samples. Water stress increased ABA levels in the ear tissue, but no treatment effects were observed for cytokinin or sugar levels (Figures 1.5, 1.6, and 1.7).

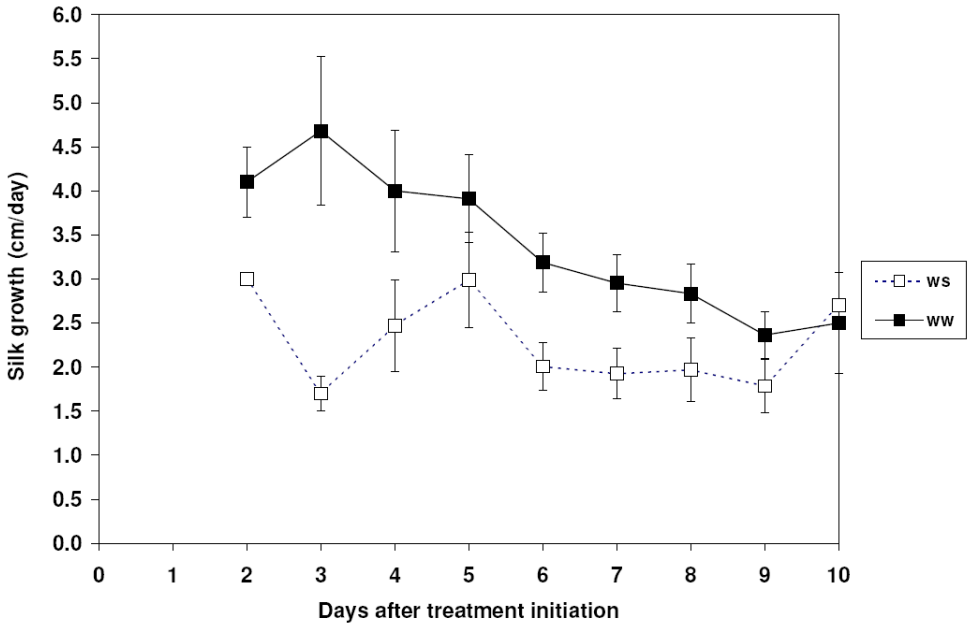


Figure 1.1. Comparison of silk growth between water treatments. Well-watered controls showed more silk growth on a daily basis compared to water-stressed plants ($p<0.01$). Means +/- SE are shown.

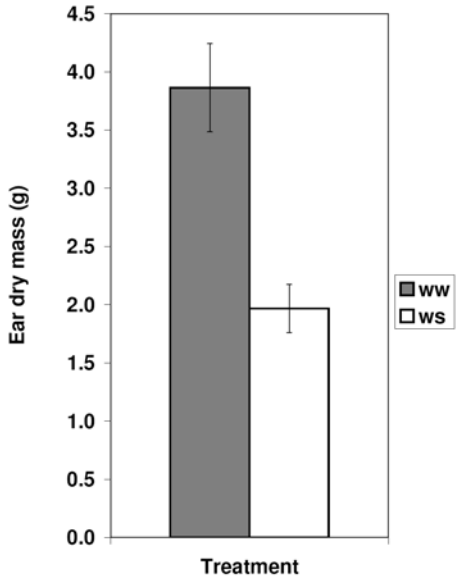


Figure 1.2. Average ear mass on a dry weight basis at the conclusion of treatments. Ears from well-watered controls had greater mass than those of water-stressed plants ($p<0.01$). Means +/- SE are shown.

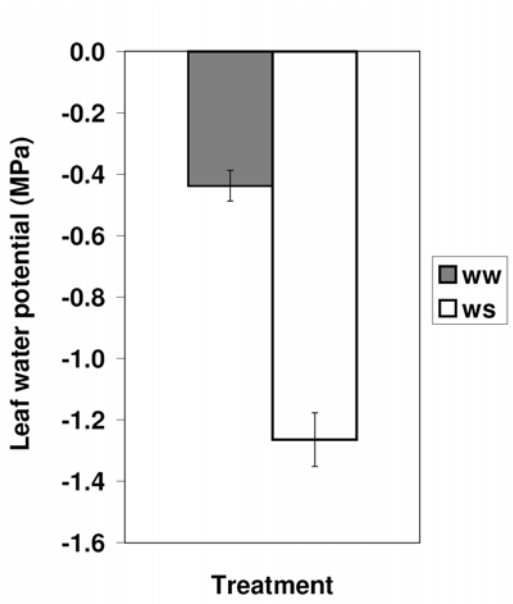


Figure 1.3. Leaf water potential measured in pre-dawn conditions using a pressure chamber. The water potential was significantly lower (more negative) for plants in the water-stress treatment compared to controls ($p<0.01$). Means +/- SE are shown.

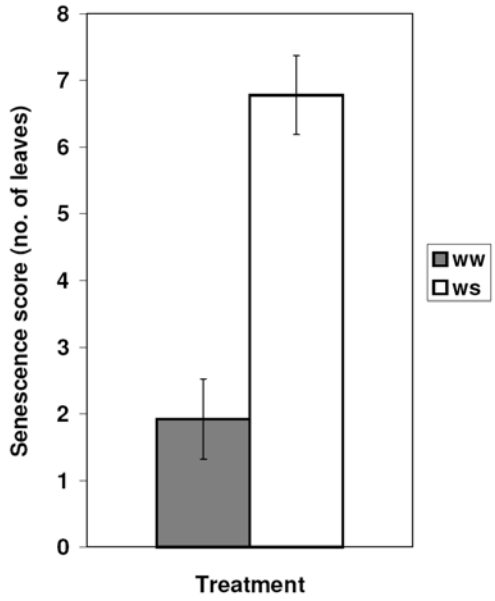


Figure 1.4. Effect of water treatment on leaf senescence measured as the total number of leaves browned on greater than fifty percent of total leaf area. Water-stressed plants showed a higher incidence of leaf senescence ($p<0.01$). Means +/- SE are shown.

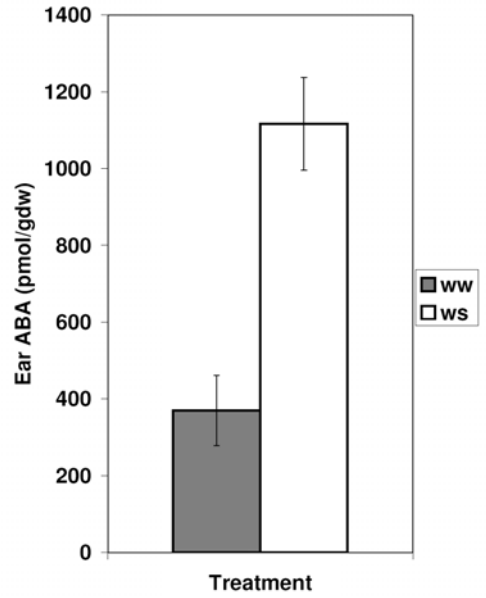


Figure 1.5. Abscisic acid levels in ear tissue. Water-stressed plants contained more ABA than controls on a dry weight basis ($p<0.01$). Means +/- SE are shown.

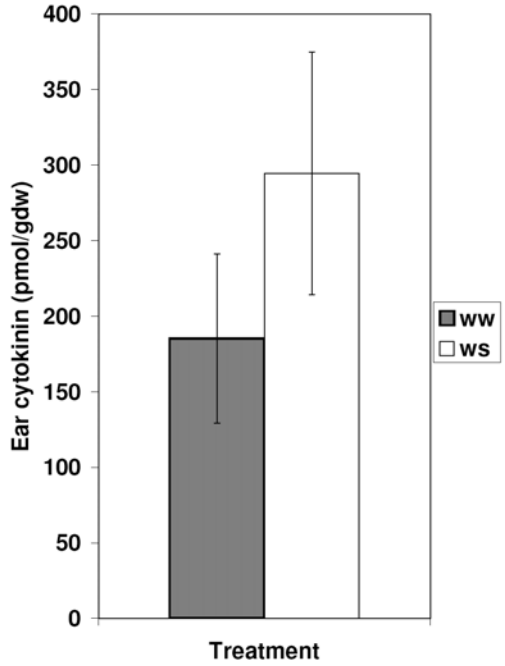


Figure 1.6. Cytokinin levels in the ear tissue expressed on a dry weight basis. No significant treatment effect was shown. Means +/- SE are shown.

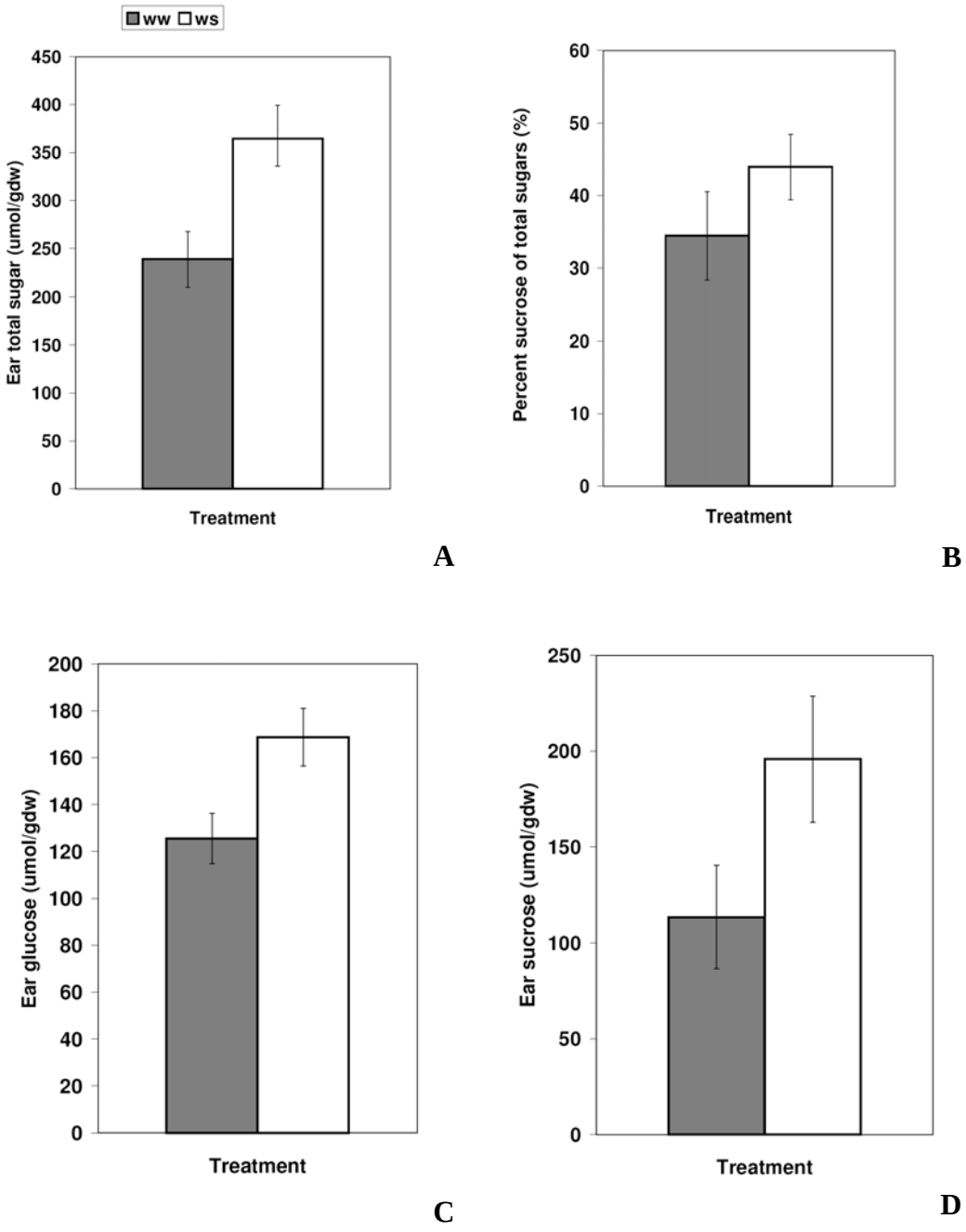


Fig 1.7a, 1.7b, 1.7c and 1.7d. Ear sugar content per gram dry weight, including total sugars, glucose, sucrose, and percent sucrose of total sugars. Total sugar (*a*) , glucose (*c*) and sucrose (*d*) concentrations were significantly higher in water-stressed ears ($p<0.01$). No significant treatment effects were observed for percent sucrose of total sugars. Means +/- SE are shown.

Experiment II

The purpose of the second experiment was to determine whether genotypes differ in their response to water deficit. Transpiration, ear growth, and silk length were measured each day during treatment imposition. Water stress reduced transpiration with no genotype x treatment effects (Figure 2.1). Daily ear growth was also reduced by water stress (Figure 2.2*a*), and the genotypes differed in their response to water deficit (Figure 2.2*b*). During water stress, silk growth was also reduced, but no genotype x treatment interaction was observed (Figure 2.3).

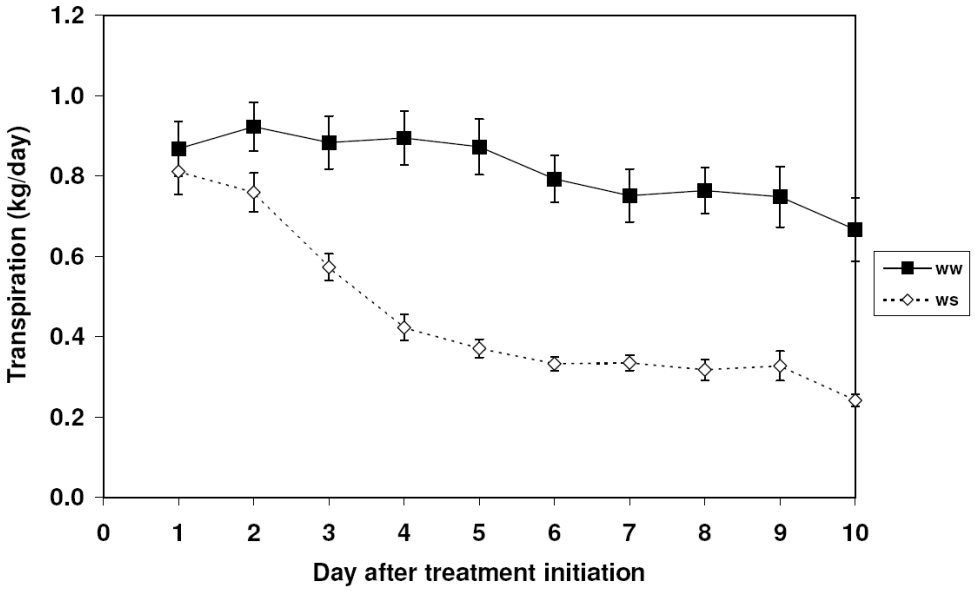
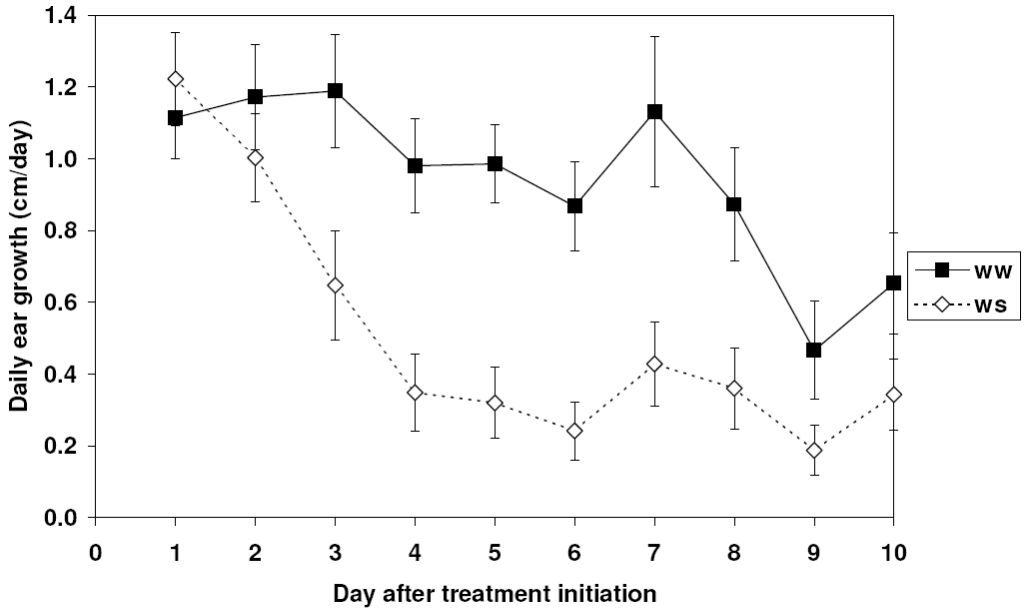
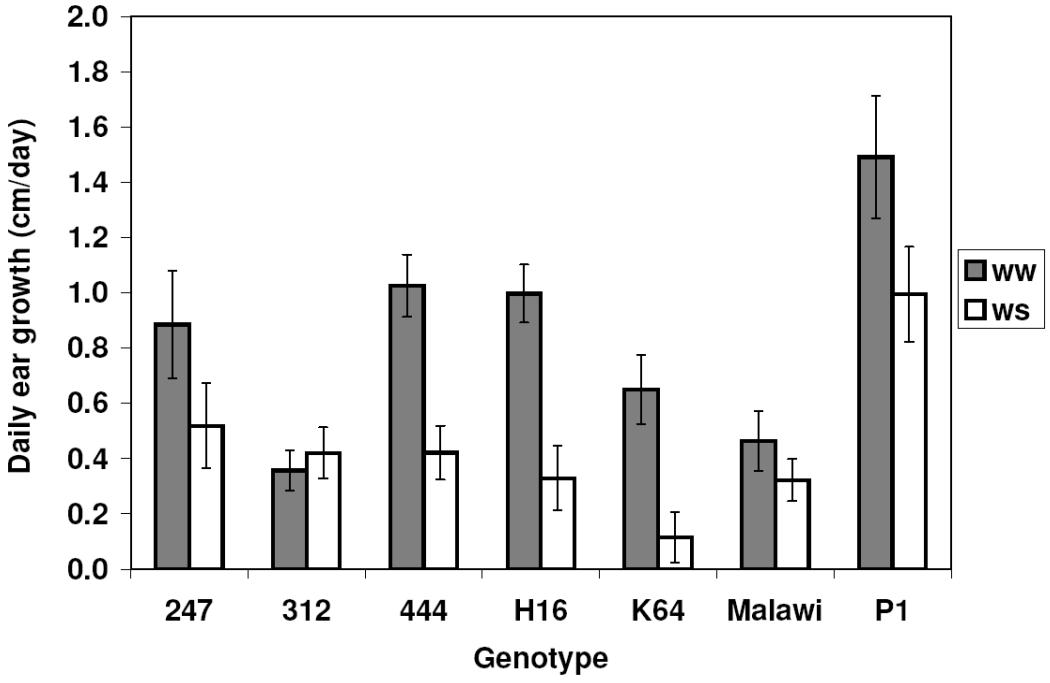


Figure 2.1. Transpiration as measured gravimetrically each morning during watering treatments. Transpiration was lowered to 0.45 kg/day on average, compared to 0.82 kg/day for control plants. A treatment effect was observed ($p<0.01$), as well as a genotype effect ($p<0.01$). A genotype x treatment effect was not shown. Means +/- SE are shown.



A



B

Figure 2.2a and 2.2b. Ear growth measured as the change in ear length each day by treatment (*a*) and genotypes (*b*). Treatment ($p<0.01$), genotype ($p<0.01$), and genotype x treatment interactions ($p<0.01$) all had an effect on daily ear growth. Means +/- SE are shown.

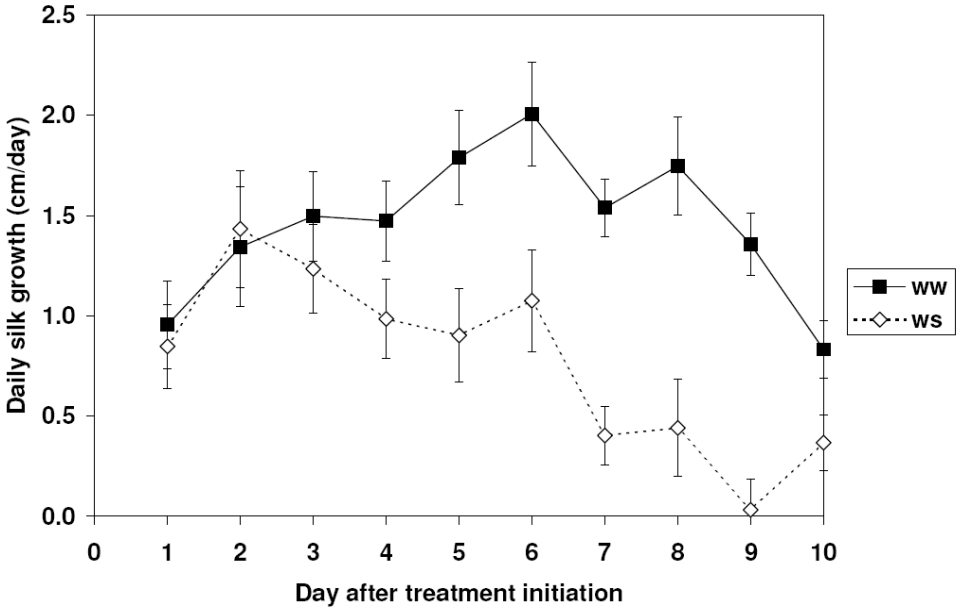


Figure 2.3. Silk growth measured as the change in silk length each day. Silk growth was greater for control plants compared to water-stressed controls ($p<0.01$) and genotypes ($p<0.01$). No genotype x treatment effect was observed. Means +/- SE are shown.

After ten days of treatment, ear dry mass, leaf water potential, and senescence were measured. Final ear dry mass was reduced under water stress and genotypes responded differently to the stress (Figure 2.4). Water stress reduced leaf water potential in comparison to controls (Figure 2.5*a*), and a genotype x treatment interaction was observed (Figure 2.5*b*). Leaf senescence was greater for plants under water-stressed conditions (2.6*a*), and genotypes differentially senesced in response to water deficit (Figure 2.6*b*).

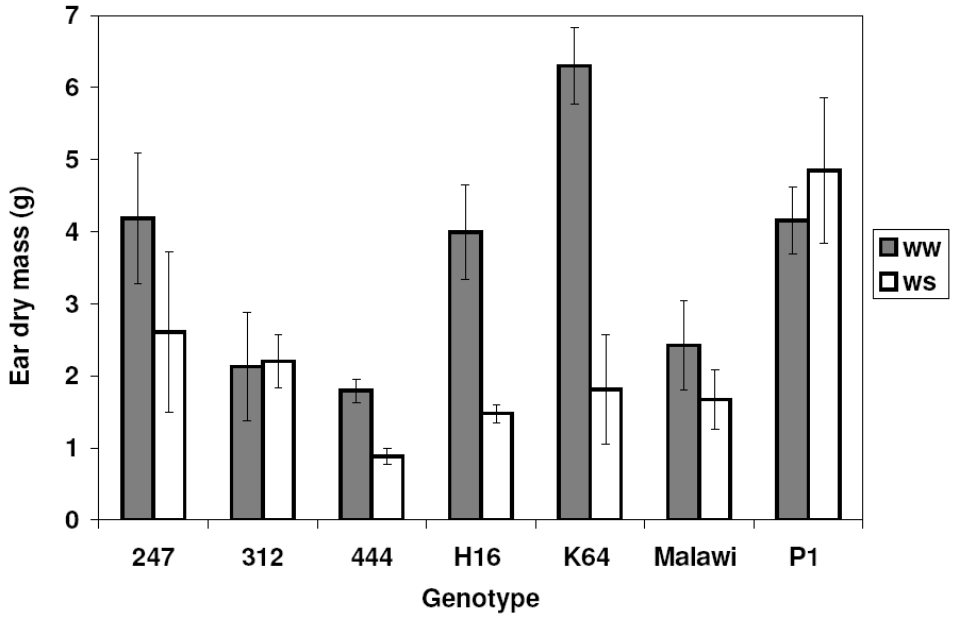


Figure 2.4. Ear dry mass at the conclusion of watering treatments. Ear dry mass was higher in well-watered controls compared to water-stressed plants ($p<0.01$). Genotype ($p<0.01$) and genotype x treatment effects on ear dry mass were also observed ($p<0.05$). Means +/- SE are shown.

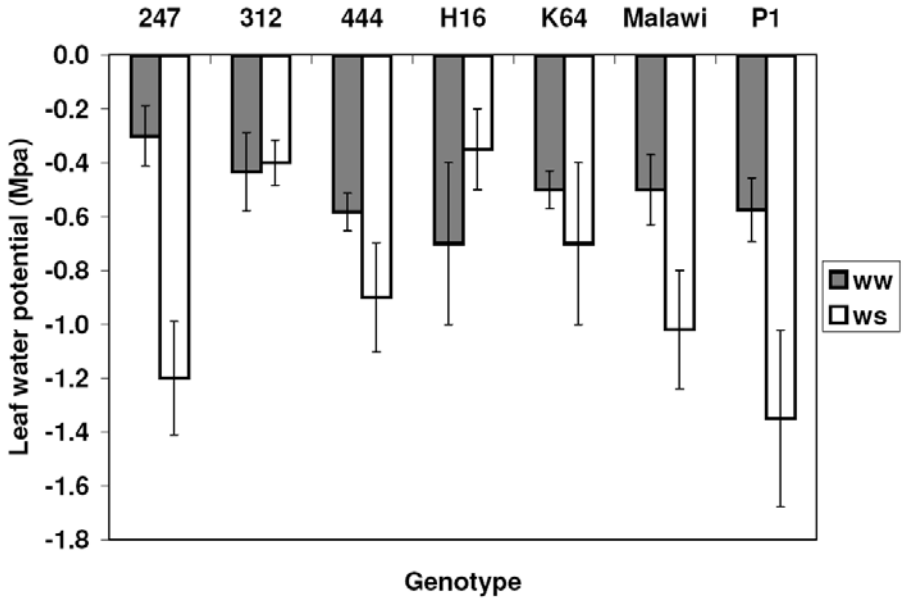


Figure 2.5a and 2.5b. Leaf water potential measured in pre-dawn conditions using a pressure chamber. The water potential was significantly lower (more negative) for plants in the water-stress treatment compared to controls ($p<0.01$). Genotypic differences were not significant, but a genotype by treatment interaction was observed ($p<0.05$). Means +/- SE are shown.

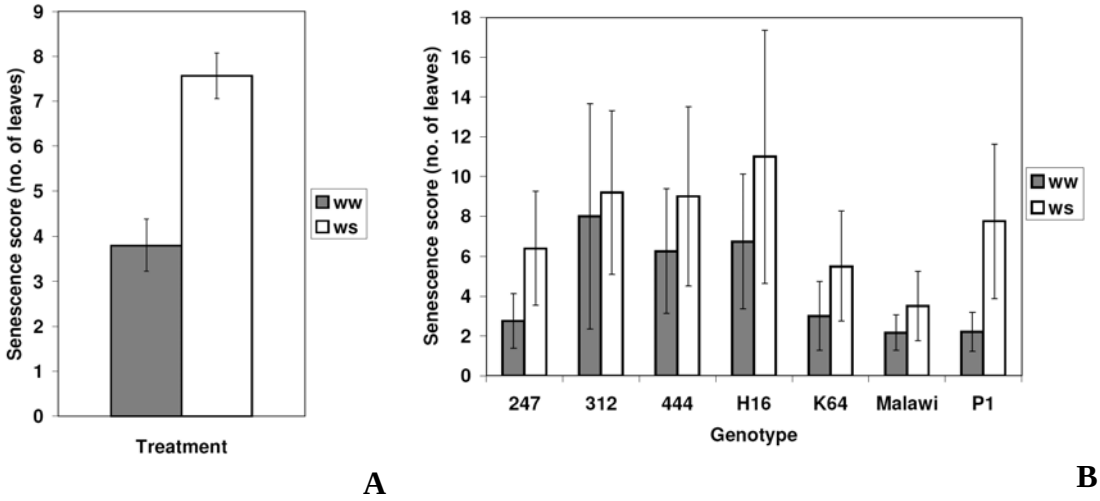


Figure 2.6a and 2.6b. Effect of water treatment on leaf senescence measured as the total number of necrotic leaves (brown on greater than fifty percent of total leaf area) by treatment (*a*) and genotype (*b*). Water-stressed plants showed a higher incidence of leaf senescence compared to controls ($p<0.01$). Differences in genotypic senescence ($p<0.01$) were also observed, but no differences in genotype x treatment response. Means +/- SE are shown.

ABA was measured in both ear tissue at the conclusion of treatments, and in leaf tissue each day during the experiment. Ears of water-stressed plants had higher ABA levels than those of the controls, but no genotype x treatment effect was observed (Figure 2.7). Leaf tissue of controls maintained low ABA levels, while water-stressed plants began to show a rise in ABA beginning with day 2 of the treatments, with a plateau after day 4 (Figure 2.8*a*). A genotype x treatment interaction also existed for leaf ABA (Figure 2.9*b*).

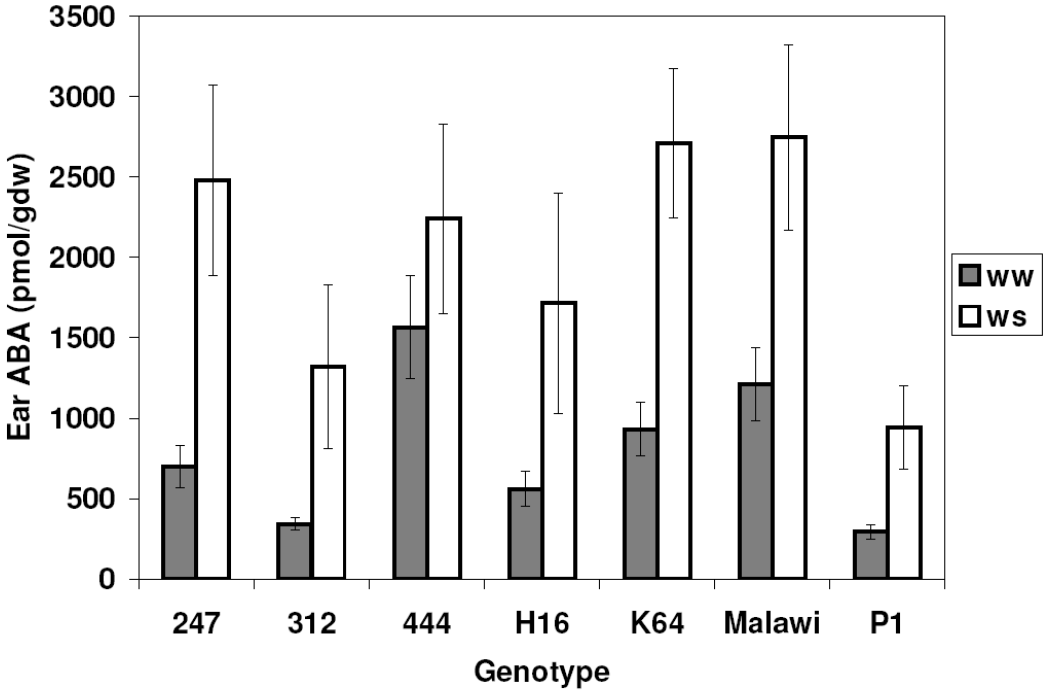
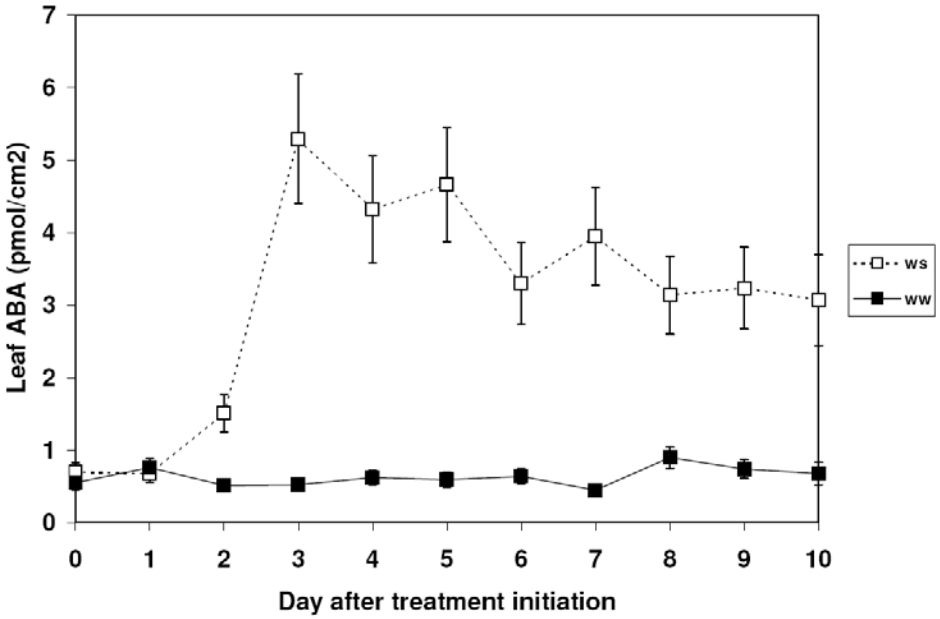
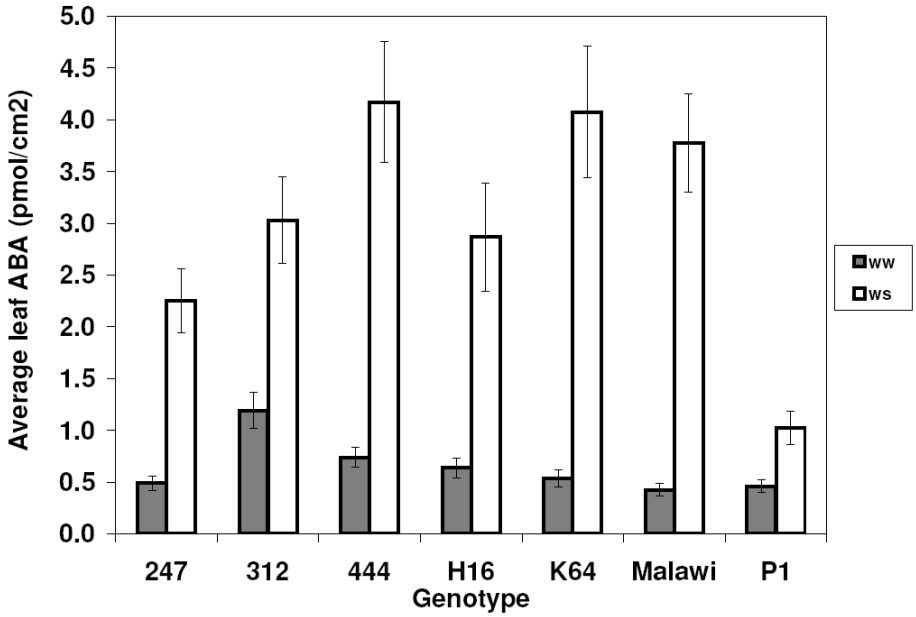


Figure 2.7. ABA levels in ear tissue at the conclusion of watering treatments. More ABA was present in ears of water-stressed plants compared to controls on a dry weight basis ($p<0.01$). Genotype effects on ear ABA levels were significant ($p<0.01$), but no genotype x treatment effect was observed. Means +/- SE are shown.



A



B

Figure 2.8a and 2.8b. Daily leaf ABA by treatment (*a*) and genotype (*b*). Water-stressed plants had higher leaf ABA levels compared to controls ($p<0.01$). Genotype ($p<0.01$) and genotype x treatment ($p<0.05$) effects on leaf ABA levels were also shown. Means +/- SE are shown.

Cytokinin concentrations were measured daily in the leaf tissue. No difference in concentration was observed between treatments or genotypes. At the conclusion of treatments, cytokinin was also measured in the ear tissue. Water-stressed plants showed higher levels of cytokinins compared to controls. Genotypes differed in cytokinin levels, but no statistically significant genotypic difference in response to water deficit was shown (Figure 2.9).

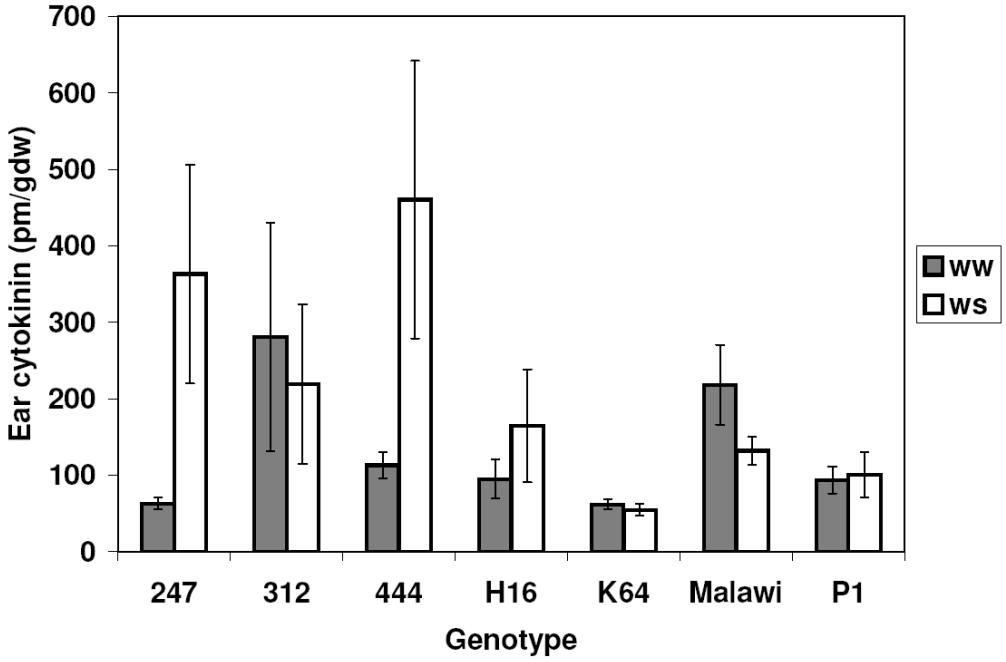


Figure 2.9 Cytokinin levels in the ear tissue at the conclusion of watering treatments. Water-stressed plants had higher ear cytokinin levels compared to controls ($p<0.01$). Genotype differences were observed ($p=0.01$), but no genotype x treatment effect. Means +/- SE are shown.

At the conclusion of treatments, sugar was measured in the ear tissue and is expressed as total sugars, glucose, sucrose, and percent sucrose of total sugars. Sugar content was also measured in the leaf tissue for each day of the experiment. A treatment effect was only shown for percent sucrose of total sugars in ear tissue (Figures 2.10, 2.11,

2.12 and 2.13), and no treatment effect was shown in the daily sugar measurements for leaf tissue (Figures 2.14, 2.15, 2.16 and 2.17).

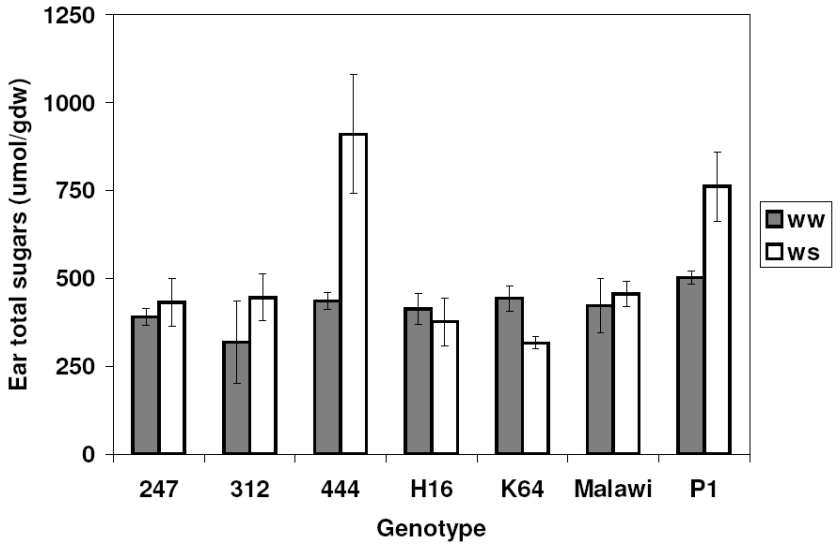


Figure 2.10. Ear total sugars measured at the conclusion of watering treatments. Differences among genotypes were observed ($p<0.01$), but no treatment or genotype x treatment effects. Means +/- SE are shown.

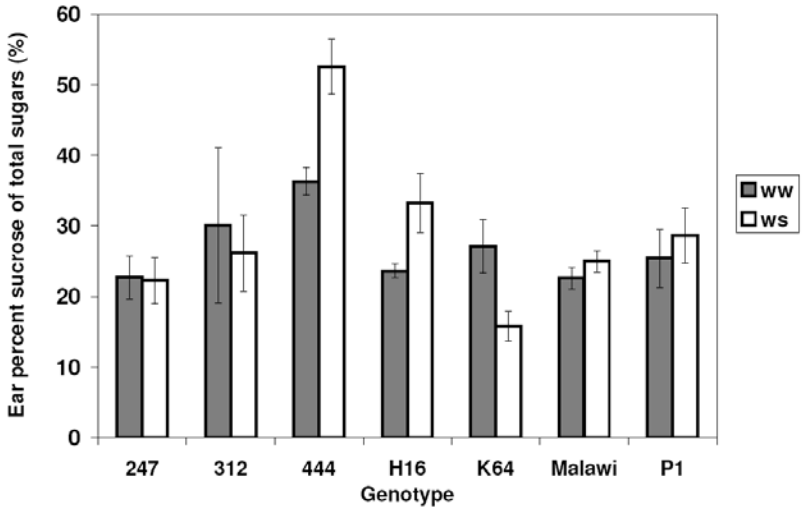


Figure 2.11. Sucrose content expressed as a percentage of total sugars in ear tissue at the conclusion of watering treatments. Ears of water-stressed plants showed a higher percentage of sucrose compared to controls ($p<0.01$). No significant genotype ($p=0.06$) or genotype x treatment effect was observed. Means +/- SE are shown.

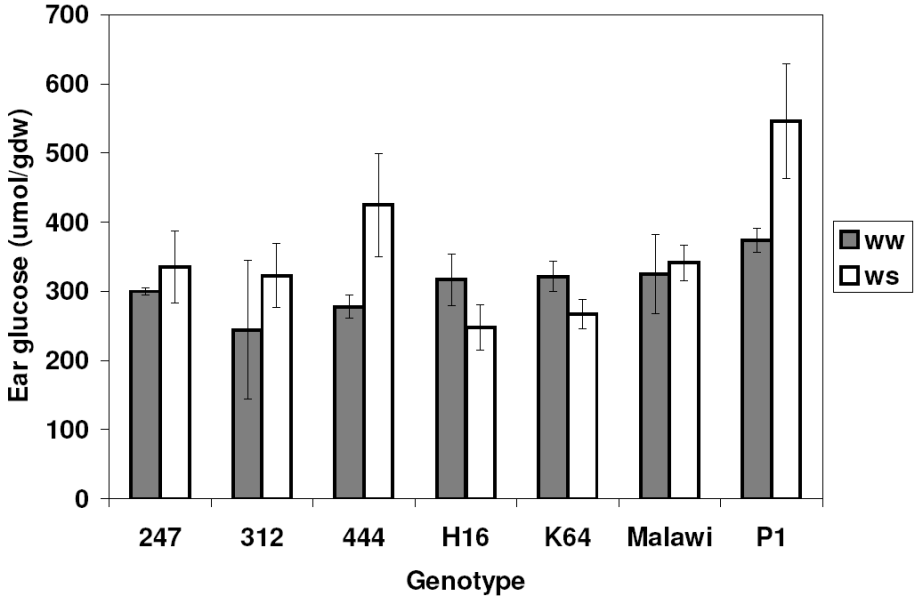


Figure 2.12. Glucose measured in ear tissue at the conclusion of watering treatments. Differences in genotypes were observed ($p<0.05$), but no treatment or genotype x treatment effects on ear glucose levels. Means +/- SE are shown.

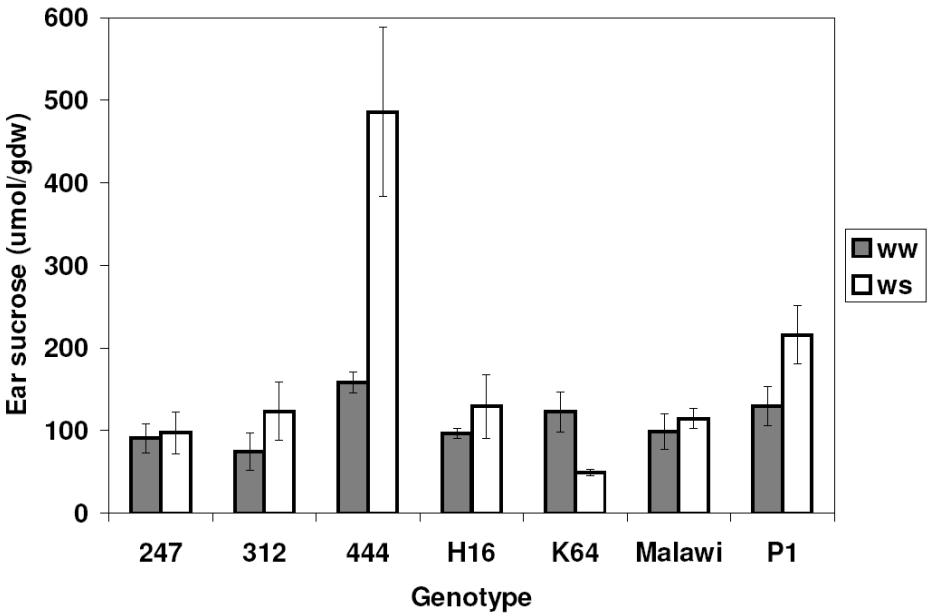


Figure 2.13. Ear sucrose levels measured in ear tissue at the conclusion of watering treatments. Genotypic differences were observed ($p<0.01$), but no treatment or genotype x treatment effects. Means +/- SE are shown.

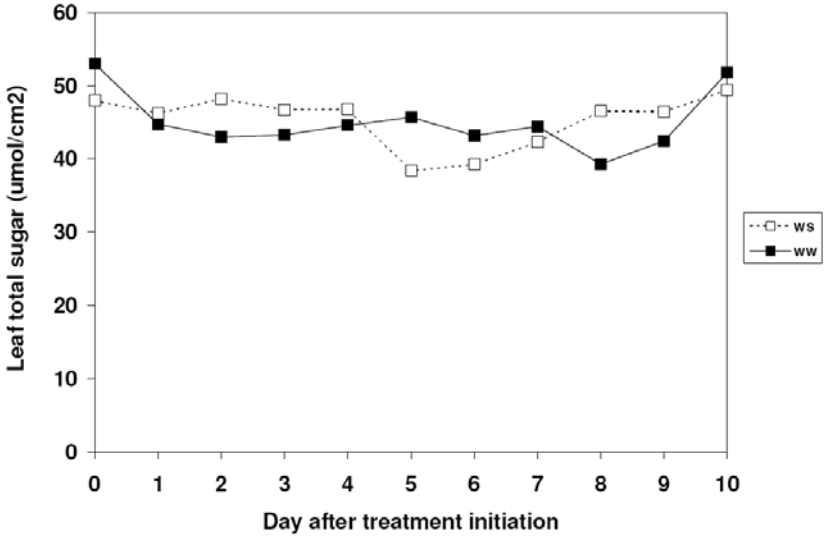


Figure 2.14. Leaf total sugars measured daily during watering treatments. Total sugars in the leaves showed no difference between treatments. A genotype effect was observed ($p<0.01$), but no genotype x treatment interaction existed. Errors bars do not exceed boundaries of symbols. Means +/- SE are shown.

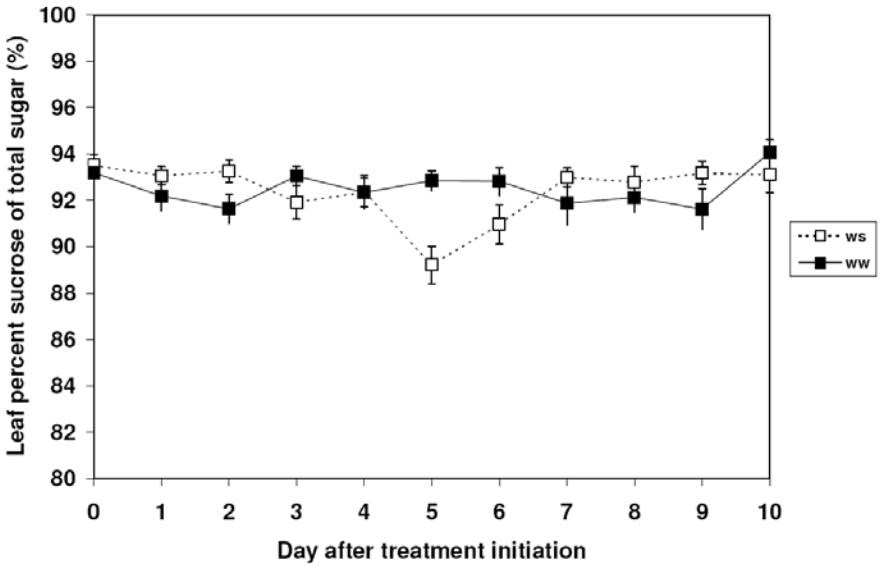


Figure 2.15. Sucrose levels in the leaves expressed as a percentage of total sugars. Samples were taken daily during watering treatments. No difference in percent sucrose was observed between watering treatments. Genotypic differences existed ($p<0.01$), but no genotype x treatment interaction. Means +/- SE are shown.

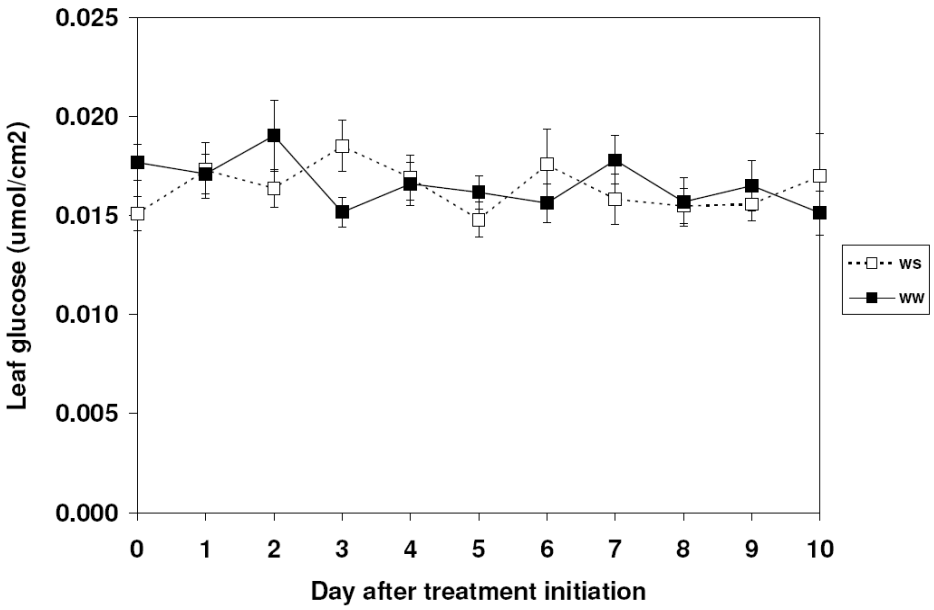


Figure 2.16. Leaf glucose levels measured daily during watering treatments. No difference was observed between watering treatments. Genotypic differences existed ($p<0.01$), but no genotype x treatment interaction. Means +/- SE are shown.

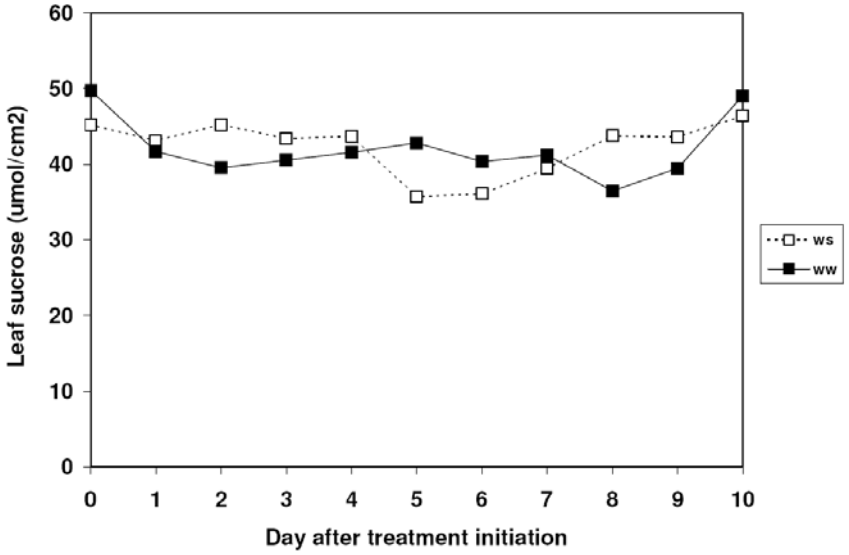


Figure 2.17. Leaf sucrose levels measured daily during watering treatments. No difference was observed between watering treatments. Genotypic differences existed ($p<0.01$), but no genotype x treatment interaction. Error bars do not exceed boundaries of symbols. Means +/- SE are shown.

In addition to sugars, starch was also quantified in the ear tissue at the treatment conclusion and daily in leaf tissue. No treatment effect was observed for starch accumulation in the ear (Figure 2.18) or leaf samples (Figure 2.19).

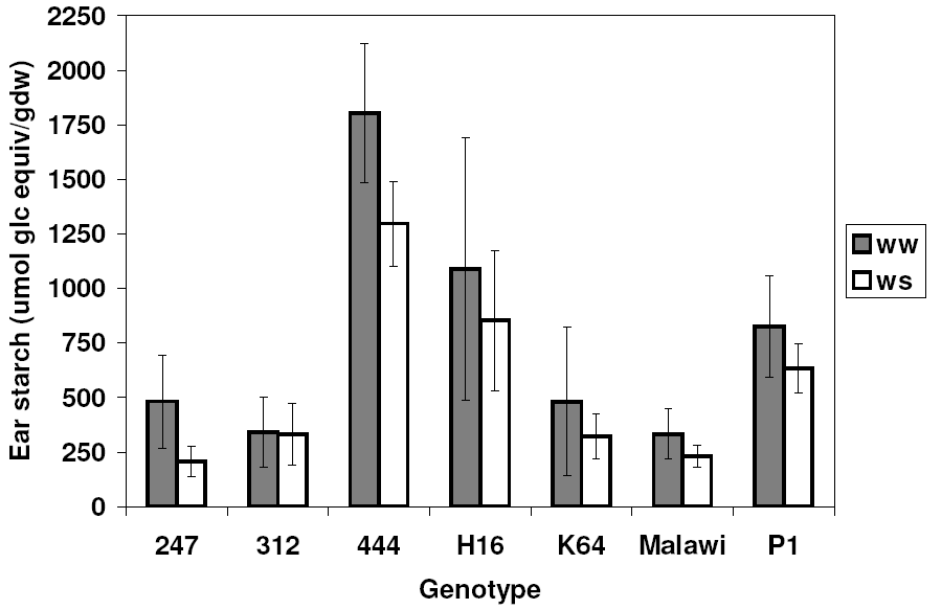


Figure 2.18. Starch levels in the ear at conclusion of watering treatments determined by cleavage into glucose. Genotypic differences were observed ($p<0.01$), but no treatment or genotype x treatment effects on ear starch levels. Means +/- SE are shown.

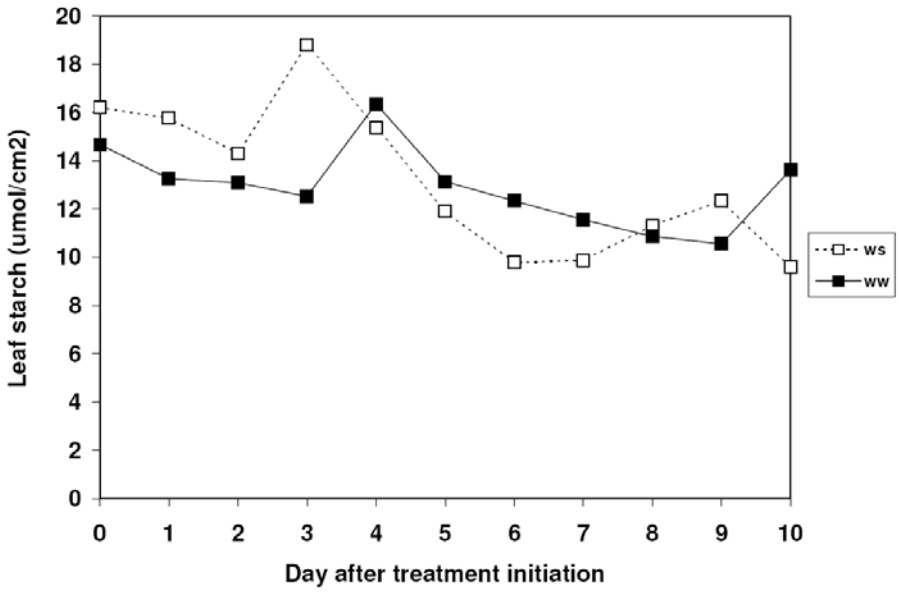


Figure 2.19. Leaf starch levels measured daily during watering treatments. No difference was observed between watering treatments. Genotypic differences existed ($p<0.01$), but no genotype x treatment interaction. Error bars do not exceed boundaries of symbols. Means +/- SE are shown.

At the conclusion of treatments, invertase was quantified by measuring activity in a timed reaction with sucrose as a substrate (data not shown). Both soluble and cell wall invertase were examined. No significant differences were observed in either invertase measured. Soluble invertase showed higher activity than the cell wall form.

Correlations among growth measurements and all laboratory quantifications were determined. The most informative results from this analysis are presented (Table 2).

Table 2. Correlations between selected traits comparing means for each genotype. $P_{0.05}$ is +/- 0.75. The dark grey shading indicates statistical significance, while the light grey coloring represents nearly significant values.

		ear dry mass		ear growth		silk growth		ABA		glucose		sucrose		starch		leaf ABA		leaf glucose		leaf sucrose		leaf starch	
		ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws
ear dry mass	ww	1.00																					
	ws	0.15	1.00																				
ear growth	ww	-0.01	0.17	1.00																			
	ws	-0.16	0.89	0.45	1.00																		
silk growth	ww	-0.22	0.35	0.73	0.60	1.00																	
	ws	-0.30	0.85	0.21	0.91	0.46	1.00																
ABA	ww	-0.17	-0.69	0.04	-0.53	-0.20	-0.75	1.00															
	ws	0.27	-0.62	-0.31	-0.78	-0.50	-0.88	0.73	1.00														
glucose	ww	0.42	0.61	0.21	0.51	0.52	0.26	-0.17	-0.10	1.00													
	ws	-0.31	0.67	0.44	0.87	0.44	0.66	-0.06	-0.50	0.41	1.00												
sucrose	ww	0.08	-0.04	0.64	0.21	0.30	-0.18	0.57	0.08	0.30	0.53	1.00											
	ws	-0.54	-0.19	0.64	0.24	0.37	0.02	0.53	-0.15	-0.17	0.55	0.76	1.00										
starch	ww	-0.29	-0.30	0.80	0.12	0.56	-0.12	0.46	-0.12	-0.05	0.30	0.76	0.89	1.00									
	ws	-0.33	-0.28	0.75	0.15	0.57	-0.06	0.38	-0.24	-0.06	0.30	0.71	0.87	0.98	1.00								
leaf ABA	ww	-0.41	-0.25	-0.16	-0.11	-0.34	0.15	-0.23	-0.41	-0.83	-0.18	-0.31	0.14	0.03	0.13	1.00							
	ws	-0.07	-0.73	-0.26	-0.61	-0.45	-0.75	0.84	0.58	-0.27	-0.21	0.44	0.36	0.28	0.29	0.06	1.00						
leaf glucose	ww	-0.35	0.73	0.19	0.74	0.36	0.92	-0.74	-0.72	0.05	0.49	-0.38	-0.06	-0.20	-0.18	0.20	-0.86	1.00					
	ws	-0.40	0.46	-0.09	0.57	0.54	0.61	-0.36	-0.59	0.48	0.43	-0.10	0.02	-0.04	0.08	-0.10	-0.23	0.37	1.00				
leaf sucrose	ww	-0.29	0.81	0.13	0.78	0.34	0.93	-0.75	-0.70	0.16	0.53	-0.37	-0.13	-0.29	-0.27	0.10	-0.86	0.99	0.42	1.00			
	ws	-0.50	0.31	-0.53	0.19	-0.28	0.47	-0.33	-0.16	-0.26	0.15	-0.66	-0.29	-0.62	-0.61	0.25	-0.36	0.63	0.23	0.65	1.00		
leaf starch	ww	0.12	0.59	0.30	0.65	0.66	0.64	-0.68	-0.80	0.54	0.29	0.00	-0.09	0.10	0.21	-0.04	-0.51	0.39	0.70	0.41	-0.22	1.00	
	ws	0.31	0.20	-0.38	0.05	-0.18	0.27	-0.71	-0.50	-0.05	-0.33	-0.50	-0.56	-0.44	-0.30	0.46	-0.24	0.13	0.27	0.14	0.02	0.58	1.00

Experiment III

The purpose of the third experiment was to compare the P1 and P2 genotypes. The P1 genotype performed well under water stress in the second experiment, and P2 was removed from analysis because of poor performance due to disease susceptibility. Each day, transpiration, ear growth and silk length were measured. Transpiration began to decline for controls around day 3 after treatment initiation, while controls maintained a higher level of water usage throughout the experiment (Figure 3.1). Ear growth was lower for water-stressed plants compared to controls, beginning with day 3 after treatment initiation (Figure 3.2). No difference in silk growth was observed between treatments or genotypes.

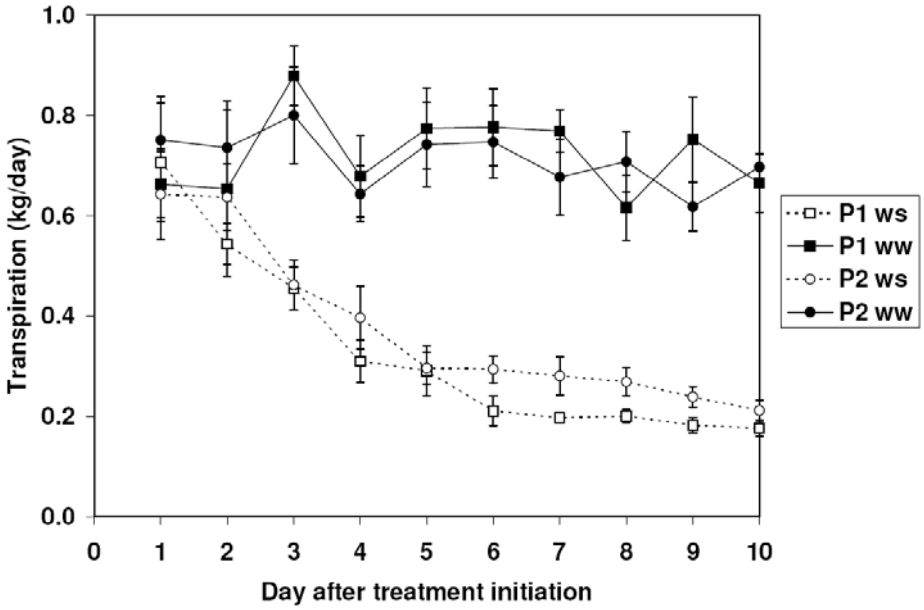


Figure 3.1. Daily transpiration as measured gravimetrically. Transpiration was significantly higher for plants under well-watered control conditions compared to plants experiencing water stress ($p<0.01$). No genotype effect was shown, but there was a significant genotype x treatment effect ($p=0.01$). Means +/- SE are shown.

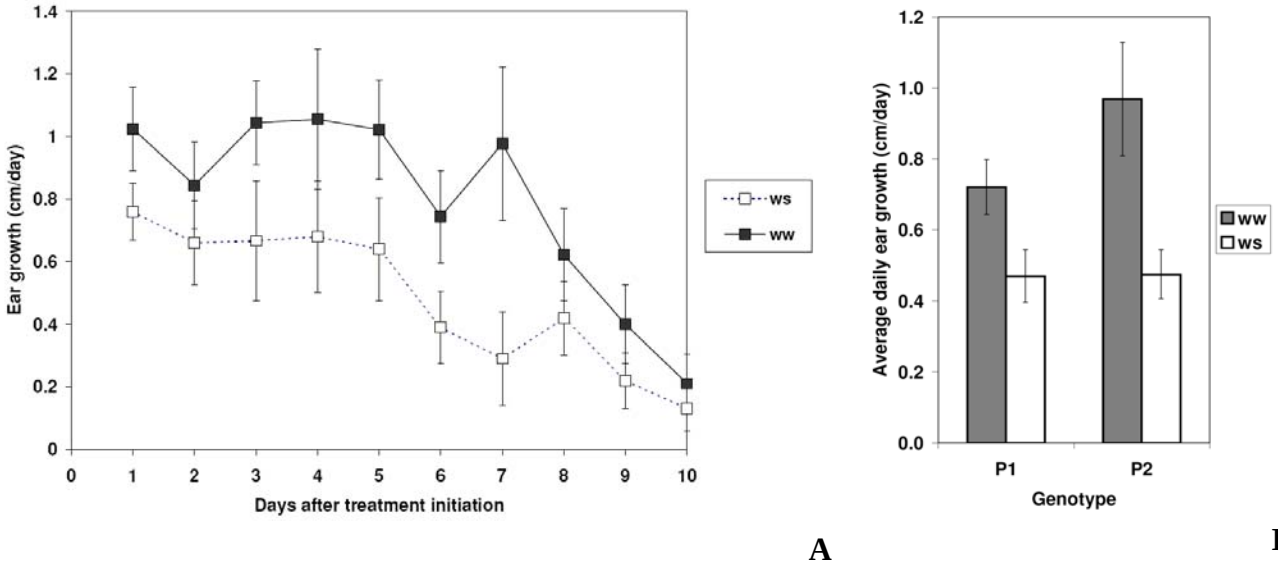


Figure 3.2a and 3.2b Daily ear growth, measured as change in ear length per day by treatment (*a*) and genotype (*b*). Growth was significantly reduced under water stressed conditions ($p<0.01$). However, no difference was apparent in the daily ear growth between the two genotypes or any significant genotype x treatment effect. Means +/- SE are shown.

After ten days of treatment, ear dry mass, leaf water potential and leaf senescence were measured. Water stress significantly lowered final ear dry mass, but no genotypic differences in response to stress were significant ($p=0.10$) (Figure 3.3). Leaf water potential was lower in water-stressed plants compared to controls, and P1 had a lower water potential than P2 in the water-stressed treatment. Controls showed similar leaf water potential (Figure 3.4). Leaf senescence was greater in water-stressed plants compared to controls, with genotypic x treatment differences not quite significant ($p=0.07$) (Figure 3.5).

Ear dry mass, kernel number, and kernel dry mass were also measured at physiological maturity (thirty days after end of treatments). Differences in these three measurements were not significant between treatments or genotypes.

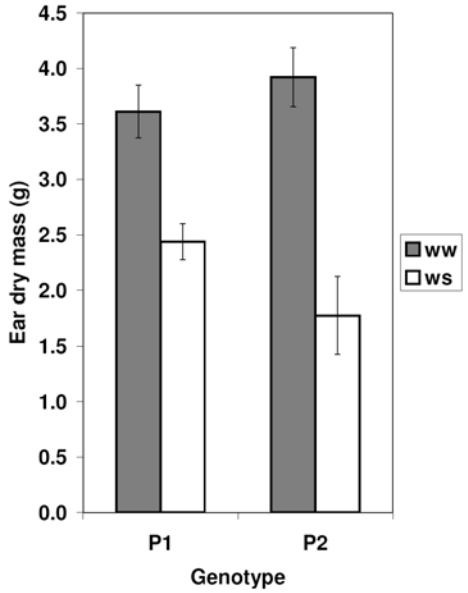


Figure 3.3. Ear dry mass at the conclusion of watering treatments. Under well-watered conditions, final ear dry mass was significantly higher than water-stressed ($p<0.01$). Differences between genotypes or genotype x treatment effects for final ear dry mass were not observed. Changes in secondary ear dry mass between treatments and genotypes were not significant. Means +/- SE are shown.

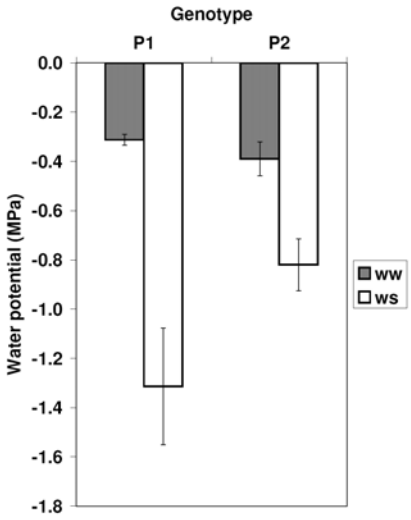


Figure 3.4 Leaf water potential measured at the conclusion of treatments. A pressure chamber was used to determine water potential under predawn conditions. Water potentials were lower (more negative) for water-stressed plants compared to controls ($p<0.01$). There was no significant genotype effect ($p<.10$), but a genotype x treatment effect was shown ($p<0.05$). Means +/- SE are shown.

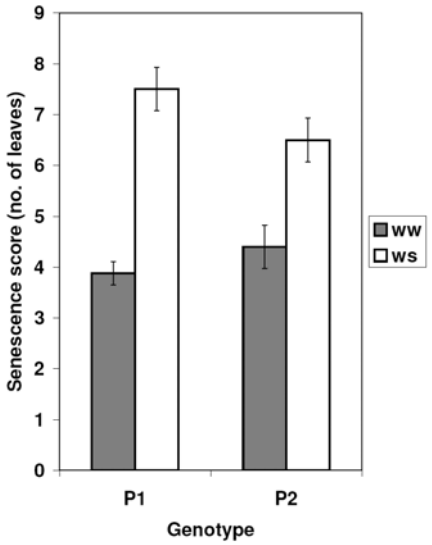


Figure 3.5. Leaf senescence was determined by counting the number of leaves with more than fifty percent browned area. The senescence score was significantly higher for water-stressed plants compared to controls ($p<0.01$). There was no significant effect shown for genotype or genotype x treatment interaction. Means +/- SE are shown.

ABA levels were quantified in the ear tissue at experiment completion. P2 showed much higher ABA levels than P1 in the water-stressed treatment, and controls showed similar levels (Figure 3.6). Water stress also increased ABA levels in the leaf tissue. P2 peaked in ABA levels sooner after treatment initiation (day 3) compared to P1 (day 5) in the low water treatment. P1 maintained slightly lower ABA levels than P2 in the control plants (Figure 3.7).

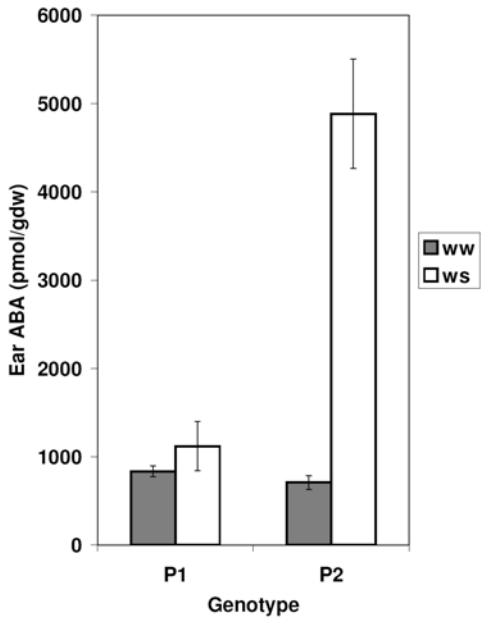


Figure 3.6. Ear abscisic acid levels measured at the conclusion of the water treatments. Effects from treatment ($p<0.01$), genotype ($p<0.01$), and genotype x treatment ($p<0.01$) were observed. Means +/- SE are shown.

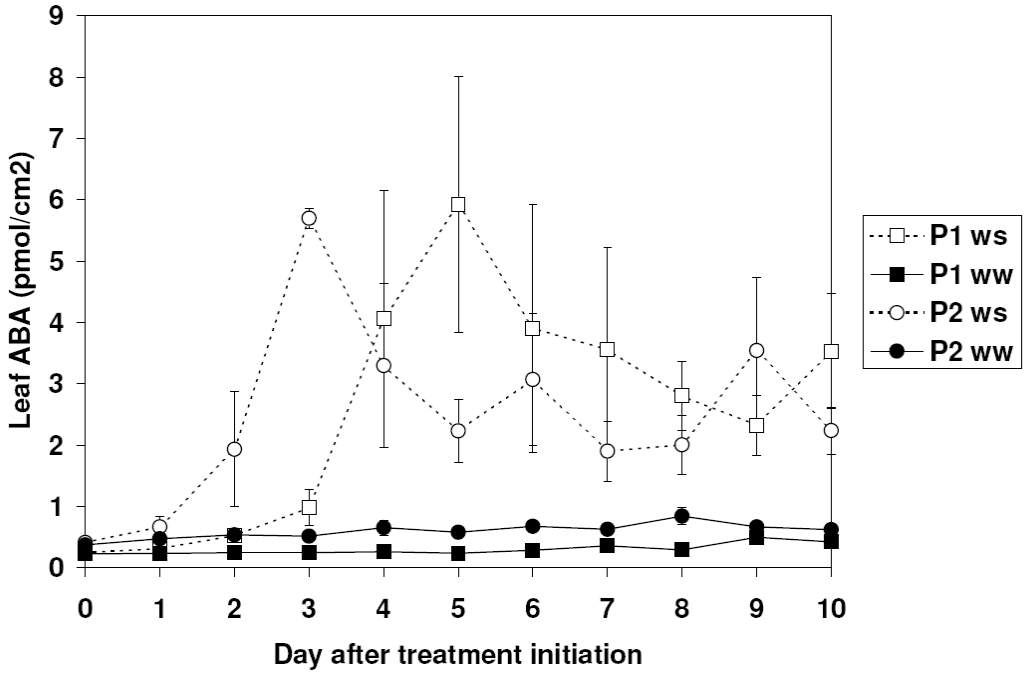


Figure 3.7. ABA levels measured daily in leaf tissue during predawn conditions. Leaf ABA concentration in water-stressed plants differed from well-watered controls ($p<0.01$). Genotype ($p<0.01$) and genotype x treatment ($p<0.01$) effects were also significant. Means +/- SE are shown.

Cytokinin measured in the ear mirrored the trend seen in ABA levels, with higher concentration seen in the water-stressed treatment and P2 showing much higher levels than P1 in low water conditions (Figure 3.8).

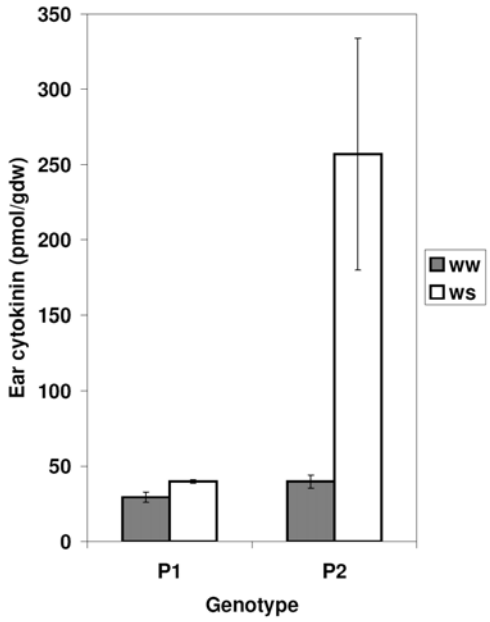


Figure 3.8. Cytokinin levels measured in the ear tissue at the conclusion of water treatments. Cytokinin concentrations were significantly higher for water-stressed plants compared to controls ($p<0.01$). Genotype ($p<0.01$) and genotype x treatment ($p<0.05$) effects were observed. Means +/- SE are shown.

Ear sugar levels after ten days of treatments showed no significant effect for treatment or genotype in total sugar or glucose levels (Figures 3.9a and 3.9b). P1 and P2 differed in sucrose content and percent sucrose of total sugars. P1 showed higher levels in the water-stressed treatment, while P2 had lowered sucrose and percent sucrose in stressed plants (Figures 3.9c and 3.9d).

Leaf total sugars increased under water-stressed conditions for P1 after day 5 of the experiment, while P2 began to show lower total sugar levels (Figure 3.10). Leaf glucose was higher in water-stressed plants, but no genotypic x treatment effect was apparent (Figure 3.11). Sucrose was also higher in P1 leaves of water-stressed plants, while P2 showed lower levels (Figure 3.12). For percent sucrose of total leaf sugars,

the water-stressed P2 plants showed a lower percent sucrose compared to P1 plants under low water conditions. The P1 water-stressed treatment showed similar percent sucrose levels to that of the controls (Figure 3.13). Sucrose comprised the majority of sugar content in the leaves.

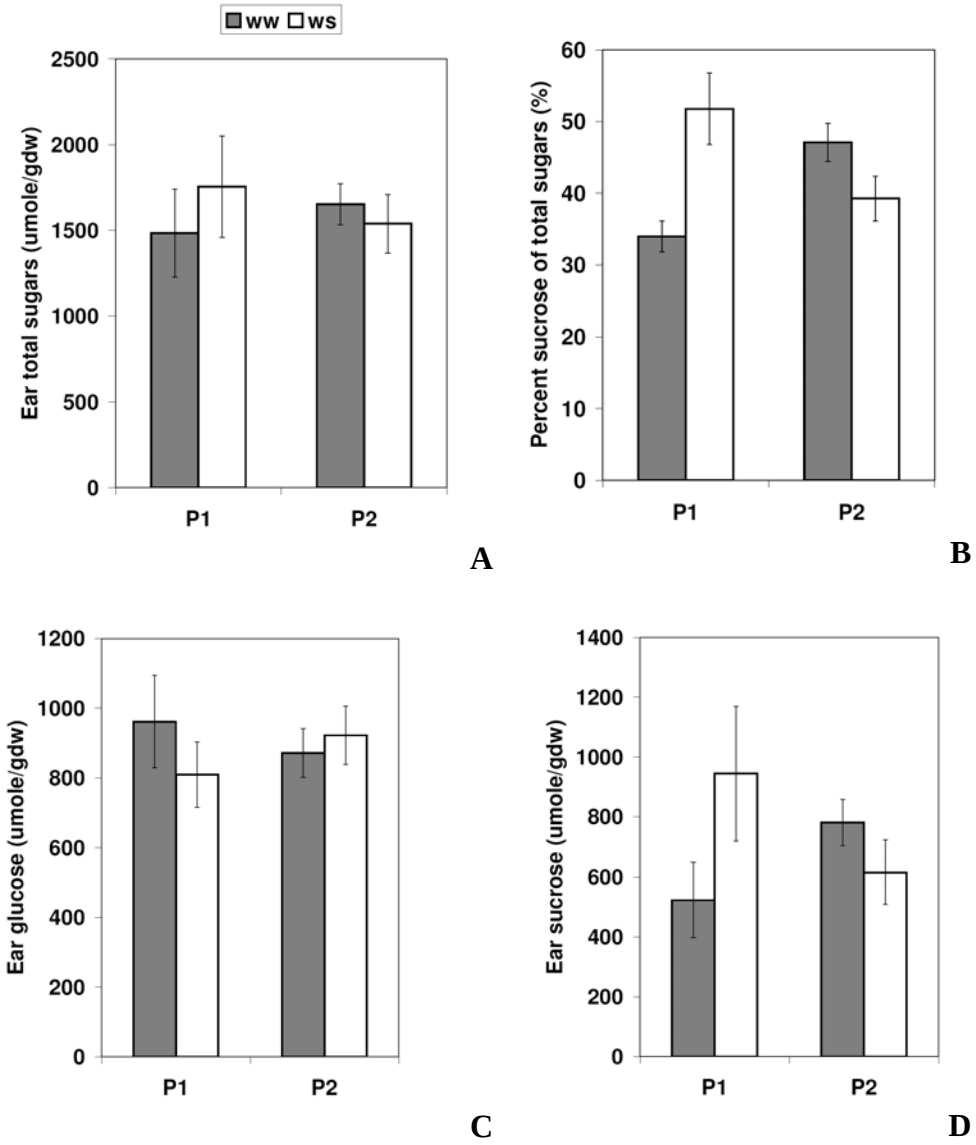


Figure 3.9a, 3.9b, 3.9c, and 3.9d. Ear sugar levels measured at the conclusion of watering treatments. Genotype is indicated on the x-axis. For total sugars and glucose levels, no treatment, genotype or genotype x treatment effect was observed. Sucrose and the percent sucrose of total sugars showed both exhibited a genotype x treatment interaction ($p<0.05$ and $p<0.01$). Means +/- SE are shown.

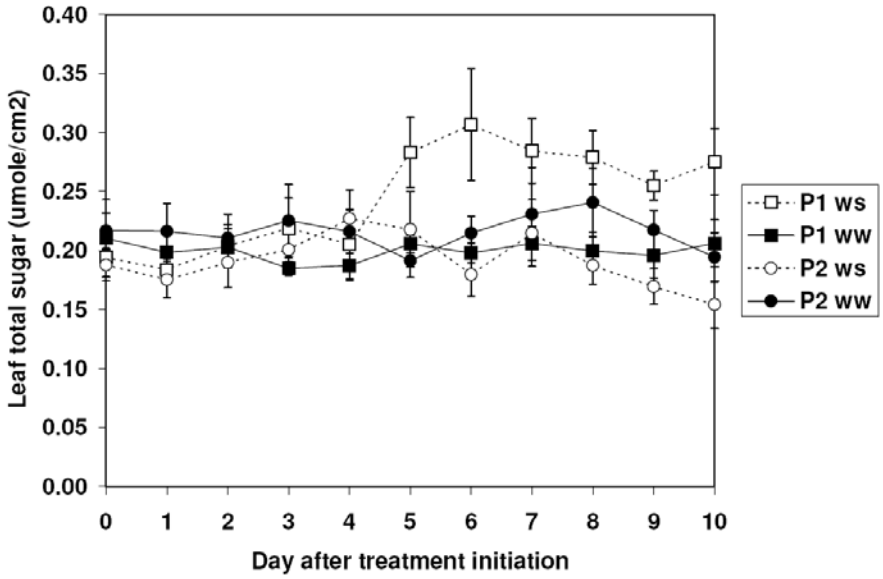


Figure 3.10. Leaf total sugars measured daily during watering treatments. Total sugars in the leaves showed a difference between treatments ($p<0.01$). No genotype effect was observed, but a genotype x treatment interaction existed ($p<0.01$). Means +/- SE are shown.

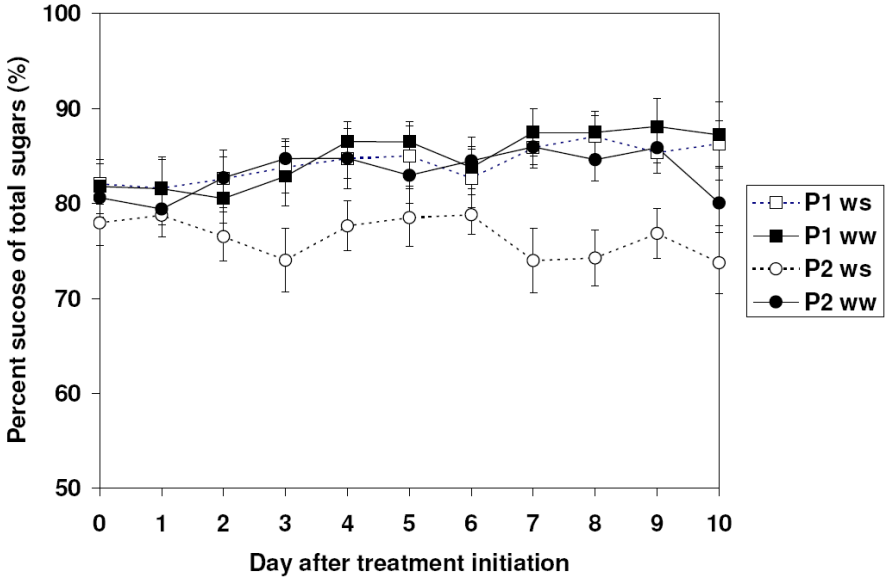


Figure 3.11. Leaf sucrose as a percentage of total sugars measured daily during watering treatments. Percent sucrose in the leaves showed significant effects for treatment ($p<0.01$), genotype ($p<0.01$), and genotype x treatment ($p<0.01$). Means +/- SE are shown.

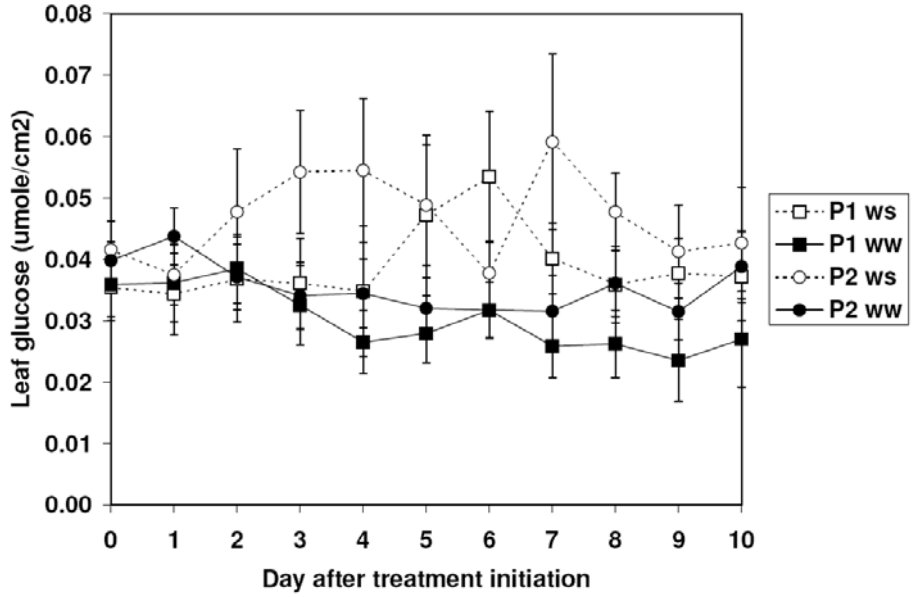


Figure 3.12. Leaf glucose levels measured daily during watering treatments. Glucose concentrations in the leaves were significantly different between treatments ($p<0.01$) and genotypes ($p<0.01$), but no genotype x treatment effect was observed. Means +/- SE are shown.

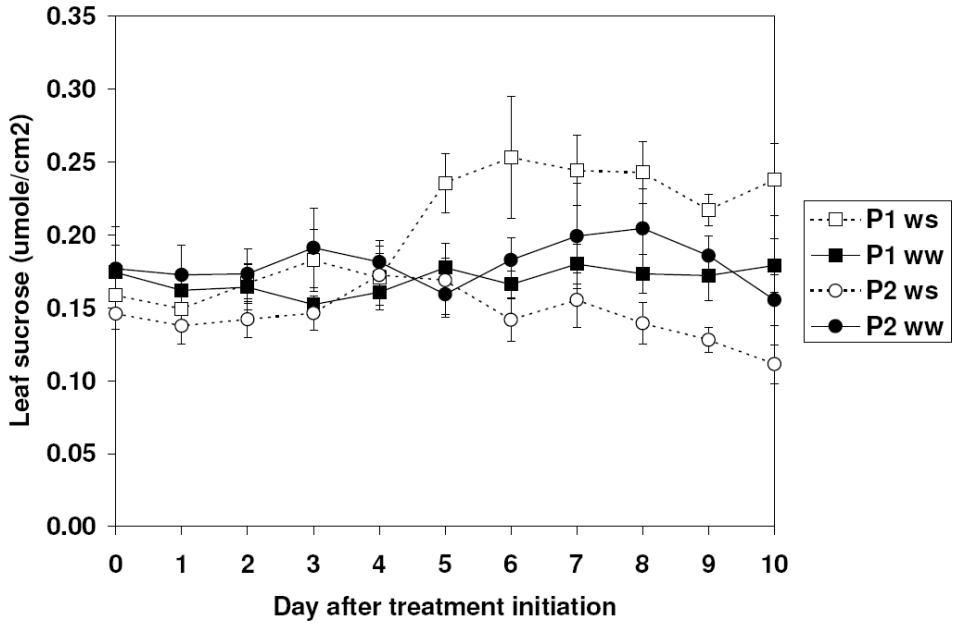


Figure 3.13. Leaf sucrose levels measured daily during watering treatments. A genotype x treatment interaction was shown ($p<0.01$). Means +/- SE are shown.

Starch levels in the ear tissue at the conclusion of treatments were similar for water-stressed plants of both genotypes. In control plants, however, P1 showed lower starch levels while P2 had extremely high starch reserves (Figure 3.14). For leaf starch levels, P2 under water stress showed a decline in starch levels earlier in the experiment (day 3) compared to P1 plants (day 7) (Figure 3.15).

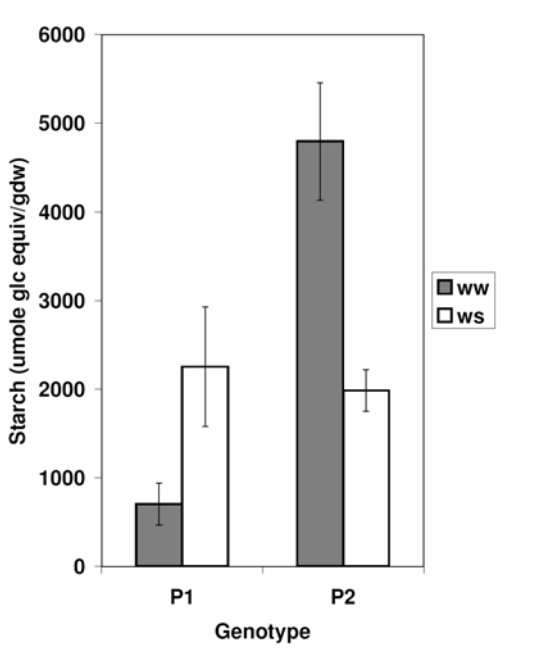


Figure 3.14. Starch in ear tissue at the conclusion of watering treatments. No treatment effect was observed, but genotype ($p<0.01$) and genotype x treatment ($p<0.01$) effects were shown. Means +/- SE are shown.

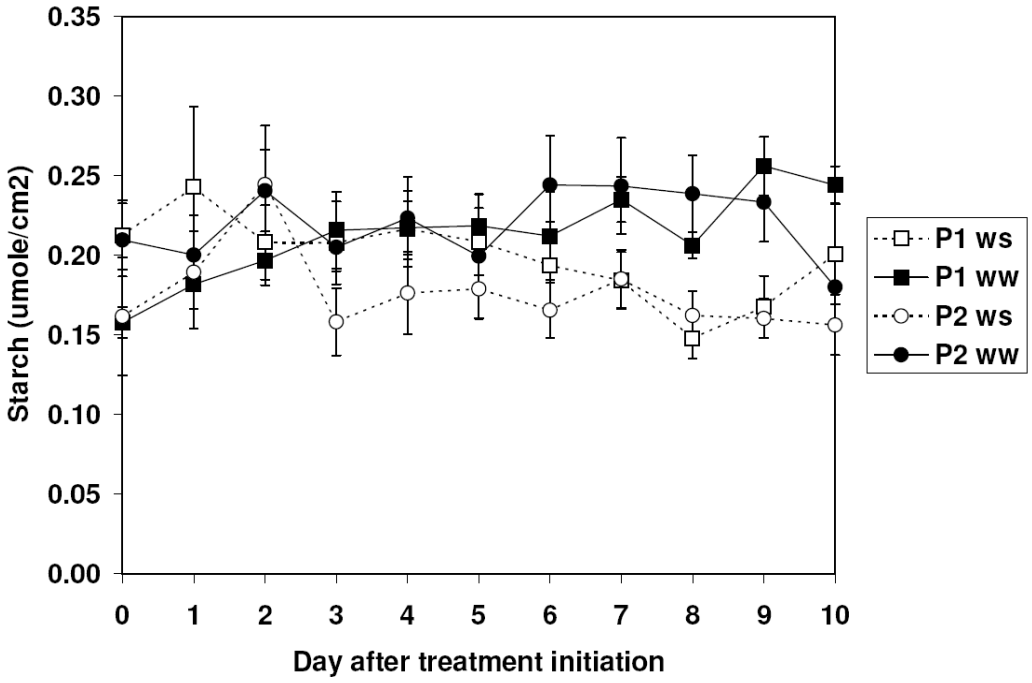


Figure 3.15. Starch measured daily in the leaf tissue during watering treatments. Leaf starch levels were not different between treatments, but a genotype effect ($p<0.01$) was observed. A genotype x treatment interaction was not quite significant ($p=0.06$). Means +/- SE are shown.

Water stress reduced invertase activity in ear tissue. When the population is divided into genotypes, P1 shows only a slight lowering of invertase activity while P2 has a more drastic decline. However, P2 had much higher activity in ears of control plants than P1 for both soluble and cell wall invertase. Cell wall invertase activity was greater than the soluble form (Figures 3.16 and 3.17).

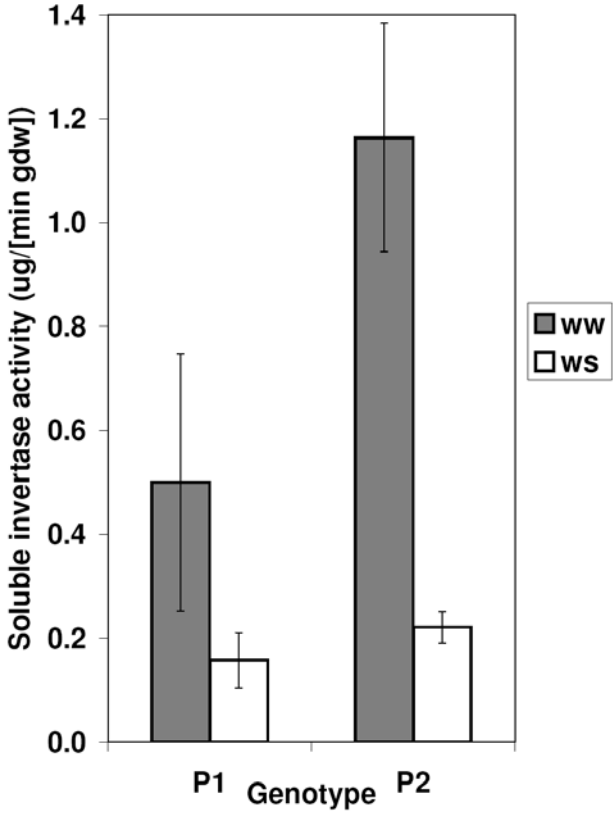


Figure 3.16. Soluble invertase activity in ear tissue at conclusion of water treatments. Activity was determined through timed reaction with sucrose solution of known concentration. Soluble invertase activity was higher in controls compared to water-stressed plants ($p<0.01$). Genotypic differences in activity were observed ($p<0.01$), but no genotype by treatment interactions. Means +/- SE are shown.

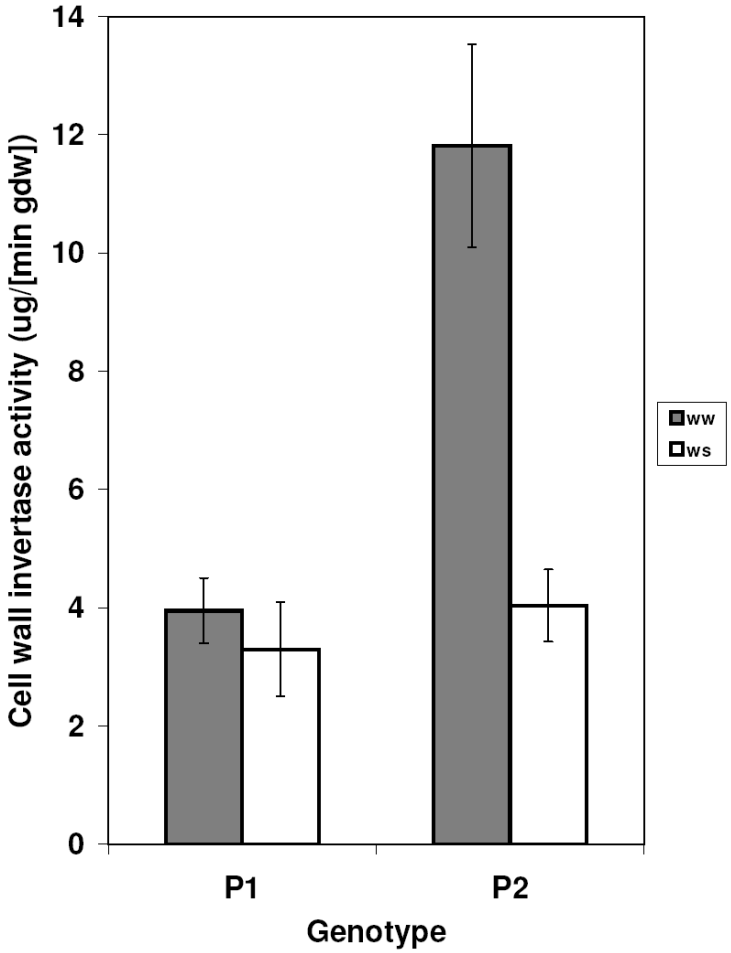


Figure 3.17. Cell wall invertase activity in the ear tissue at conclusion of watering treatments. Activity was determined through a timed reaction with a sucrose solution of known concentration, after performing dialysis to remove native sugars present in the tissue. Well-watered controls showed higher cell wall invertase activity compared to water-stress plants ($p<0.01$). Genotypic differences were observed ($p<0.01$) and a genotype x treatment effect ($p<0.04$). Means +/- SE are shown.

Interpretations of results are discussed in the following chapter.

CHAPTER 4

DISCUSSION

Treatment Effects on Transpiration and Growth

Between experiments, transpiration rates were consistent for both well-watered and water-stressed treatments (Figures 1.1, 2.1 and 3.1). This indicates that although different watering techniques were utilized, the stress intensity imposed upon the plants in the low water treatment was comparable between experiments. Leaf ABA levels also rose in response to water stress, while well-watered controls maintained low leaf ABA levels. ABA began to increase around the same day transpiration began to decline in water-stressed plants, suggesting the hormone's role in closing stomata, which regulates transpiration. The abrupt decline in transpiration rate is most likely due to stomatal closure in response to water potential declining below a threshold.

Daily ear growth corresponded closely to transpiration rates for plants of both treatments. Controls maintained higher ear growth rates, while water-stressed plants began to decline in ear growth around one day after transpiration was reduced. In general, growth began to decline for both treatments as the experiment progressed and the plants aged. Silk growth rate also declined for water-stressed plants in the first two experiments. This decline in silk growth rate imparts a delay in silk emergence (longer anthesis-silking interval) formerly reported in plants under drought stress (Monneveux & Ribaut, 2006). Water deficit also lowered ear dry mass at the end of stress treatments in all experiments, confirming that the stress imposed was sufficient to alter reproductive growth.

Hormones

Abscisic acid levels were significantly higher in ears of water-stressed plants, similar to previous reports in the literature (Zinselmeier *et al*., 1999; Setter *et al*., 2001). Boyer & McLaughlin (2007) suggest that in many published reports where ABA in ears is expressed on a fresh-weight basis, the increase in ABA is due to the decrease in the tissue water content during drought episodes, not due to net synthesis and accumulation of ABA. However, the data presented from this experiment do not support this interpretation, as ABA concentrations are expressed on a unit dry weight (ear) or area (leaf) basis. Also, Asch *et al*. (2001) showed no difference in ovary ABA concentration when expressed on dry weight basis under field conditions. Different drought tolerance mechanisms may be utilized by plants in a field setting compared to greenhouse conditions, such as increase rooting depth, and could account for this disparity in ABA levels between water-stressed plants of this study and those of Asch *et al*.

As growth slowed under water stressed conditions, ear cytokinin levels increased. Cytokinin trends actually mirrored those of ABA. Because cytokinin is a growth promoter, decreased levels are expected under drought conditions. In post-pollination maize ovaries, cytokinin concentrations peak at the same time as cell division (Lur & Setter, 1993). In addition, Brugiere *et al*. (2008) found that a gene involved in coding for cytokinin synthesis shows increased expression in developing post-pollination kernels. The decrease in cell division expected in aborting kernels suggests that cytokinin concentrations would decrease in developing maize kernels experiencing water stress. Surprisingly, the opposite was observed in the current study. Previous studies have reported that during water deficit, cytokinin concentrations in post-pollination maize kernels and pre-pollination ear tissues remain similar to those of

controls (Ober *et al*., 2001; Setter *et al*., 2001) and the hormone is antagonistic to ABA (Davies *et al*., 2005). ABA and drought conditions also stimulate the expression of cytokinin oxidase, the enzyme that degrades cytokinin (Brugiere *et al*., 2003). Pospisilova *et al*. (2005) reported increased cytokinin levels in water-stressed leaf tissue of various plants, including maize. These authors found that exogenous ABA treatment increased cytokinin levels in maize leaves, and as ABA levels rose during water stress more cytokinin was produced, perhaps to counteract some of ABA's effects. This type of concurrent hormonal augmentation is consistent with the data presented in the ear tissue of water-stressed plants for this study. However, the mechanism appears to be complicated and more evidence would need to be examined before making any firm conclusions.

Carbohydrate Partitioning

In the leaves, more invertase activity increases the hexose:sucrose ratio in the leaves during water stress (Trouverie & Prioul, 2006). In the last experiment of this study, glucose levels were higher in water-stressed leaves. Due to the decrease in photosynthesis that occurs when a plant is water-stressed, sugar levels might be expected to decrease in leaf tissue. However, water stress did not reduce leaf total sugars in any of the experiments of this study. Furthermore, Trouverie *et al*. (2003) found *elevated* hexose and sucrose levels in leaf tissue in response to water stress and suggest an enhancing effect of ABA upon invertase activity. Starch may be a source of the sugars present, and in the third experiment a decrease in leaf starch was observed.

During water stress, the opposite shift in hexose:sucrose has been observed in the kernels – a reduction in invertase activity lessens and decreases hexose relative to sucrose (Trouverie *et al*., 2003). This suggests that during stress a signal for decreased

invertase expression is present in the ovaries but not in leaves. Furthermore, different invertase genes exist that have different tissue and stress response specificity. Previously, it was thought that converting sucrose to hexoses in leaves would slow down export. But ^{14}C labeling studies show an increase of fixed carbon exported during stress, so differential activity of invertase at each end of the phloem apparently helps regulate export efficiency and increase the gradient of osmotic potential to create stronger sinks (Trouverie & Prioul, 2006).

In Exp. II, higher percent sucrose of total sugars was observed in the ovaries of water-stressed plants. Given that drought stress decreases expression of invertase in kernels and therefore invertase-mediated sucrose cleavage, these results are consistent with earlier findings of Zinselmeier *et al*. (1995b) that water stress decreases invertase activity. However, the expected decrease of ear glucose levels due to lowered invertase activity was not observed during the current experiment. When sucrose is cleaved to hexoses, a shift occurs in osmotic potential (twice as many osmotically active solutes after cleavage) – (Trouverie & Prioul, 2006). Stem infusions of sucrose have been found to restore ovary water potential and turgor (Zinselmeier *et al*., 1999). Also, Zinselmeier *et al*. (1995a) found that sucrose appeared to be more important than other osmotically-active solutes, such amino acids and salts, in sustained maize reproductive growth under water stress. Maize genotypes selected for high osmotic adjustment (OA) had higher yield than low-OA genotypes in drought conditions (Chimenti *et al*., 2006). In contrast, Bolanos and Edmeades (1991) reported that tropical maize lines usually have low osmotic adjustment and selection for improved yield during drought did not show a relationship between yield performance and OA. It appears that that sucrose cleavage is an important step for drought response in the current experiment. Correlations with reproductive growth will be discussed later.

No significant treatment effect was observed in ear starch levels. This was not an expected result, as previous studies have shown that starch reserves are utilized under water-stressed conditions due to the reduction in phloem-imported photosynthate products (Zinselmeier et al., 1995b; Trouverie & Prioul, 2006). However, Andersen et al. (2002) actually found elevated starch levels in ovaries of drought-stressed maize plants. It does not appear that starch is a limiting factor for early-aborting kernels. Leaf starch levels did appear to decline in water-stressed plants, seen most clearly in the third experiment. This most likely indicates remobilization of the carbohydrates in the absence of photosynthesis due to demands of the growing ear tissue under water stress.

No significant trends were seen in invertase activity in the ears for Exp. II. Complications encountered during this first attempt at quantifying invertase activity made it difficult to see any sharp trends in the data. However, a general decline in invertase activity under water-stressed conditions was observed, prompting another examination. In Exp. III, the analysis proceeded more smoothly, and water-stress clearly reduced invertase activity levels in the ear tissue for both soluble and cell wall invertase. This result corresponds to former experiments examining invertase activity under water stress (Zinselmeier *et al*., 1999). Overall, cell wall invertase activity (40-140 ug/[min gdw]) was higher than soluble invertase (0.5-3.5 ug/[min gdw]). Andersen et al. (2002) concluded that soluble invertase is more important in early stages of development, while cell wall invertase activity increases later in kernel growth. However, cell wall invertase appears to be important in pre-pollination ovaries as well (Zinselmeier *et al*., 1999; McLaughlin & Boyer, 2004). In addition, Carlson and Chourey (1999) assert that solubility is affected by sample handling techniques

and much of the activity attributed to soluble invertase in the ovaries is actually cell wall invertase mistaken for the soluble form during laboratory analysis. They found that the soluble form is more important in other organs such as the roots, while cell wall invertase is most important in the endosperm tissue in post-pollination maize kernels. ABA has also been shown to increase expression of soluble invertase in leaf tissue (Trouverie *et al*., 2003), but this upregulation caused by ABA has not been seen in ear invertase activity (Andersen *et al*., 2002). The findings presented here are consistent with those results.

Genotypic Differences in Behavior

Experiment II

Genotypic variations were seen in ear growth rates and final ear dry mass. Malawi and 312 had little change in ear growth between treatments, but had the lowest growth rates in control conditions. P1 had the highest growth rate under both water-stressed and well-watered conditions. The genotypes 247, 312, Malawi and P1 all had little change in final ear dry mass for the water stress treatment compared to control plants, and K64, 444, and H16 all had a substantial decline in final ear dry mass under water stress. Because of the variation in reproductive growth under water stress, this provides a good setting for comparisons of traits among genotypes and their relationship to ear development.

When looking at the correlations between genotypic averages for all traits measured, a few important observations are apparent. Ear and leaf ABA in water-stressed treatments were negatively correlated with final ear dry mass, daily ear growth and daily silk growth. This supports the idea that yield potential is higher for lines that maintain low ABA levels under water-stressed conditions. Also, the final mass and

growth measurements were positively associated with average glucose concentrations during water stress, suggesting that invertase-mediated sucrose hydrolysis is a key step in reproductive growth processes under drought conditions.

Interestingly, among the seven genotypes in Exp. II and two genotypes in Exp. III, final ear mass and growth measurements in water-stressed plants were positively associated with leaf carbohydrate concentrations in well-watered plants. Accordingly, constitutive osmotic accumulation (high levels of carbohydrates acting as solutes) in the leaves may be important for growth performance under water stress. In addition, leaf carbohydrates in well-watered plants were negatively associated with ABA concentrations in water-stressed leaves. In other words, genotypes possessing leaves with higher ABA levels during water stress episodes had fewer carbohydrates when water was available. This is consistent with the idea that a genotype's behavior in drought episodes may be associated with its constitutive solute accumulation.

Experiment III

Under water-stressed conditions, P1 had a greater ear dry mass than P2 although the differences were not quite significant ($p=0.10$). Both genotypes behaved similarly when well-watered. No differences were seen in daily ear or silk growth, so ear dry mass at the conclusion of the experiment appears to be the best method for assessing drought response for Exp. III. In breeding trials conducted at CIMMYT, P1 has shown superior drought tolerance compared to P2 in field conditions (Ribaut *et al*., 1996).

The P1 genotype had slightly lower transpiration than P2 plants under water deficit, and the P1 leaf water potential was much lower (-1.3 MPa) than that of P2 (-0.8 MPa) for the water-stressed plants. The two genotypes also differed in their senescence

response. Well-watered plants had similar numbers of senesced leaves for both genotypes, but P1 had more senesced leaves than P2 in the water-stressed treatment. Despite these indications that P1 may have experienced a more severe water stress, the final ear dry mass for P1 was actually higher than that of the P2 genotype.

The ears of the P1 genotype showed much lower ABA and cytokinin levels in the water-stressed treatment than P2. P1 also appeared to have a more delayed water stress response, showing peak leaf ABA levels at day 5, compared to day 3 for P2 water-stressed plants. This suggests that a more conservative strategy for eliciting a hormonal stress response via ABA is more advantageous for reproductive success.

P1 showed higher levels of sucrose and percent sucrose of total sugars in the leaves, while the P2 genotype showed a decline in leaf sucrose and percent sucrose of total sugars under water stress. P1 may have maintained higher sugars by adjusting its solute concentration, decreasing phloem export or sustaining a higher rate of photosynthesis. P1 showed higher starch in ears of stressed plants than P2, evidence that P1 is also not utilizing its emergency reserves of nonstructural carbohydrates during water stress. Levels of invertase activity in the ear tissue were similar for both genotypes in water-stressed conditions. P2 experienced a more drastic reduction in invertase activity, however, as the enzyme's activity in control conditions was much higher in P2 than in P1.

Conclusions and Future Research Directions

Using ear growth rate and final ear dry mass as a yield performance indicator, P1 appears to perform the best of all genotypes surveyed under water-stressed conditions in this particular experimental setting. Physiologically, P1 maintains low transpiration,

low water potential, greater leaf senescence, low ear ABA and cytokinin, delayed ABA response in the leaves, more sucrose and percent sucrose in the leaves and ears, more starch in the ear tissue, and little change in ovary invertase activity. Physiologically, invertase-mediated sucrose cleavage seems to be a key step in carbohydrate flux to the developing ovary under water stress. This enzyme was down-regulated in water-stressed conditions and behaved differently between the two genotypes in Exp. III. Identifying the differences in invertase behavior and their relationship to yield performance in several genotypes would be a crucial next step.

To expand upon these experiments, I would first attempt three modifications to the current procedure: 1) determination of yield at physiological maturity, 2) more sampling points for ear tissue and 3) more effort in determining solute potential. A better indication of yield at physiological maturity would strengthen the findings of this experiment. In Exp. III, yield was measured at 30 days after pollination. However, pollination was delayed until after water treatments had ended and a majority of the ears were barren due to aging silks or florets. An experimental design that would allow treatment imposition during the flowering period and a typical pollination schedule would be ideal for this type of yield estimate. Also, more frequent sampling over the ten-day treatments for the ear tissue would be informative (instead of one final measurement). This increase in sampling would allow a more holistic understanding of the temporal behavior of hormones, carbohydrates and invertases during water stress. To test osmotic hypotheses, I would invest more effort in determining solute potential and relative water content. Although solute potential measurements were attempted using a psychrometer for experiments I and II, the data was too noisy to see any clear trend. In future work, I would work on optimizing this instrument or utilize another method.

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